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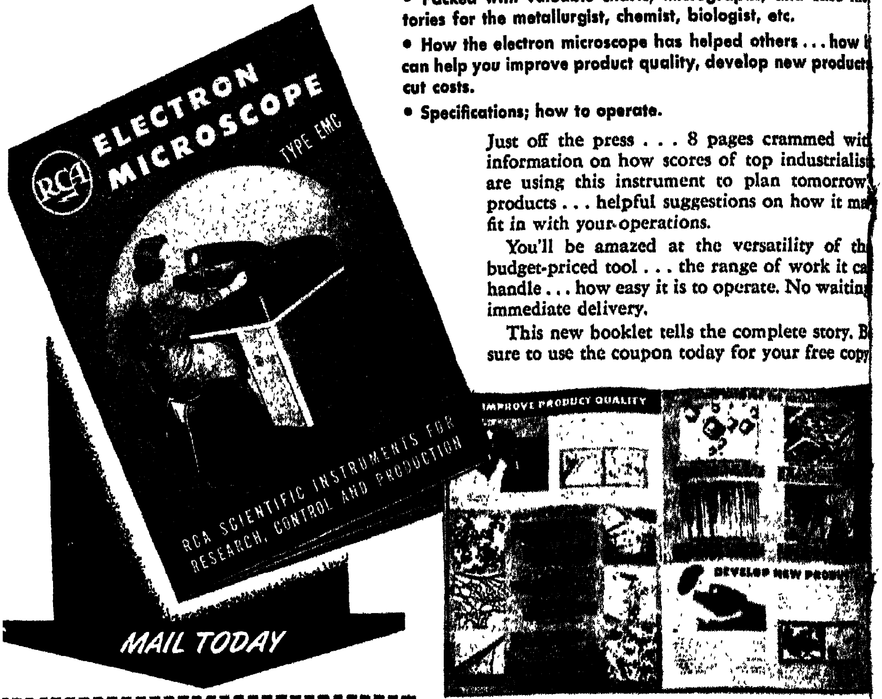
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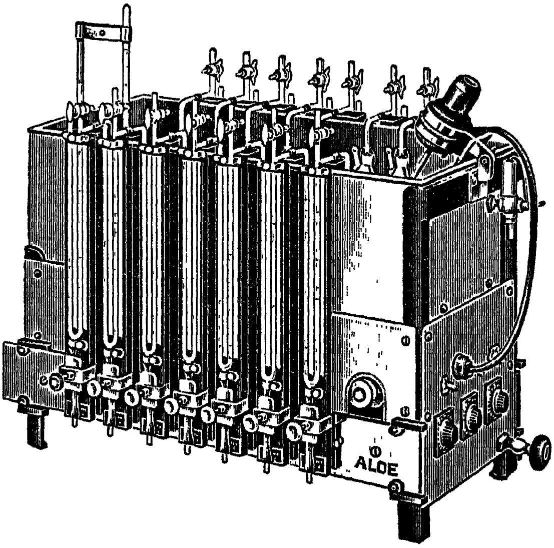

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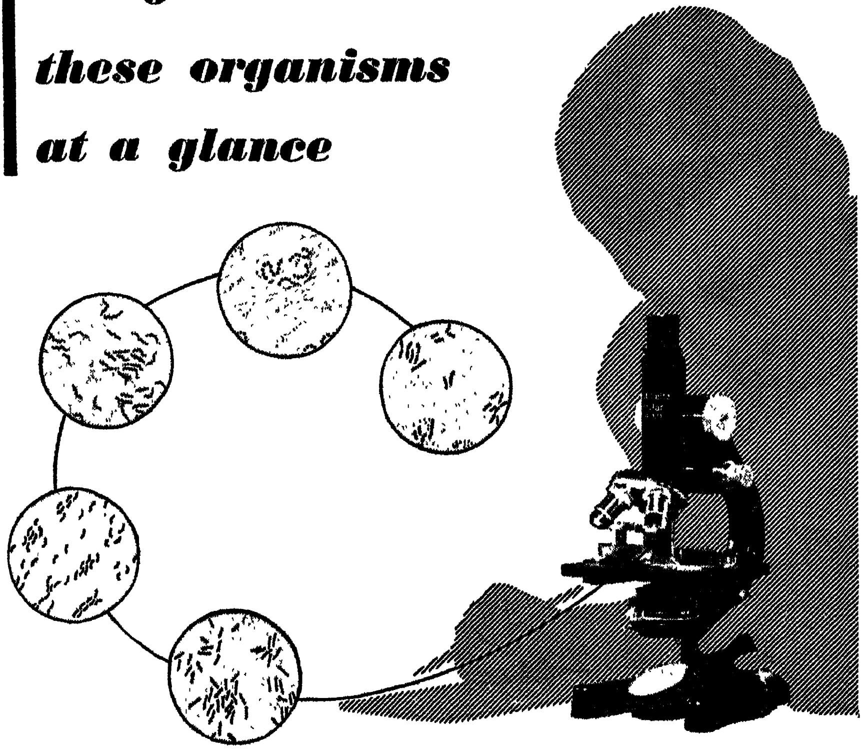

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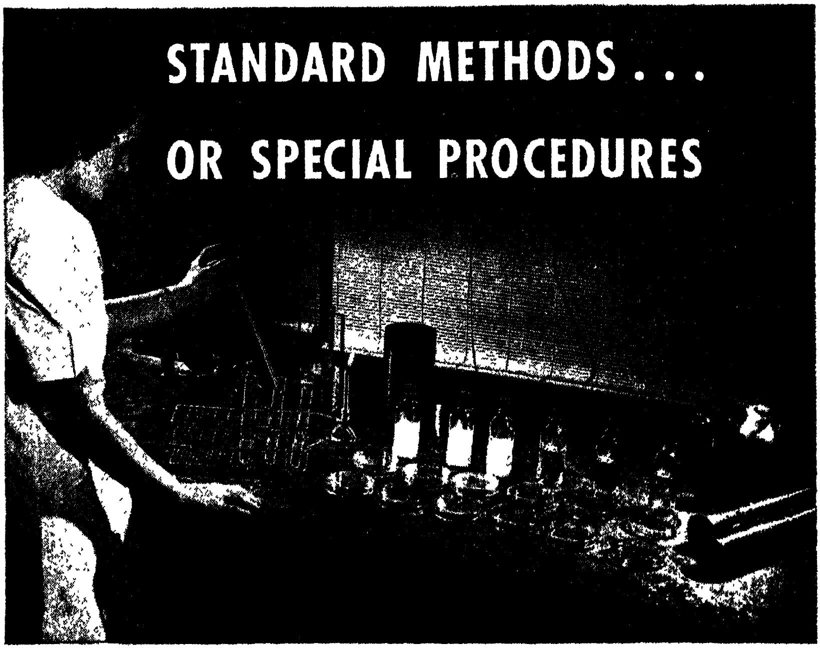

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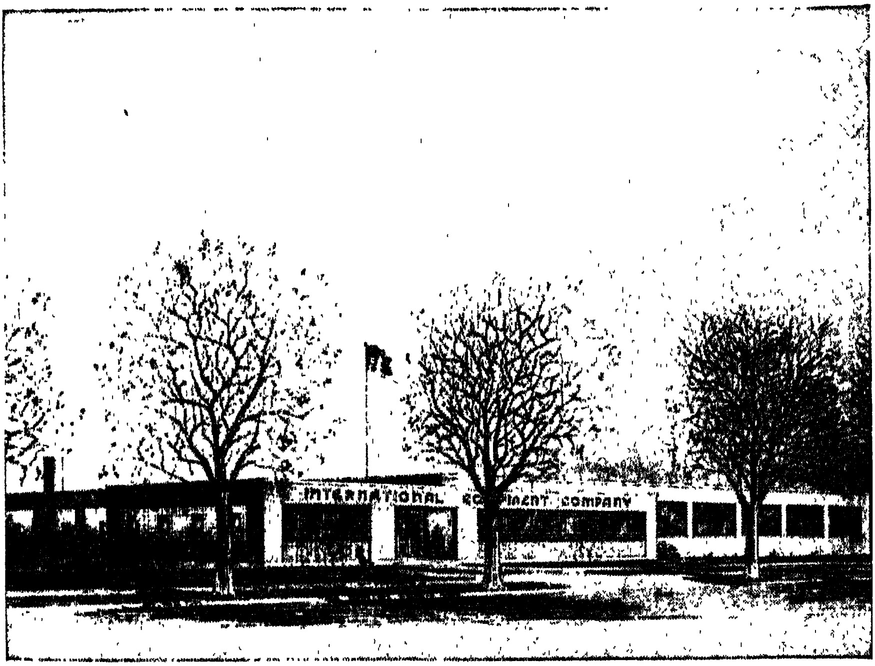

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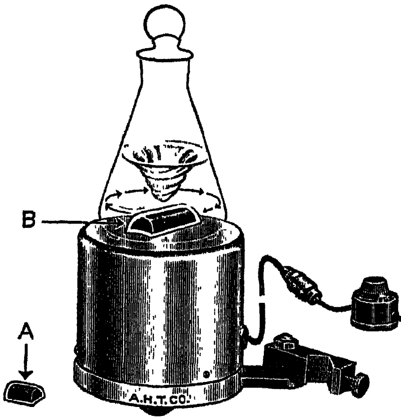

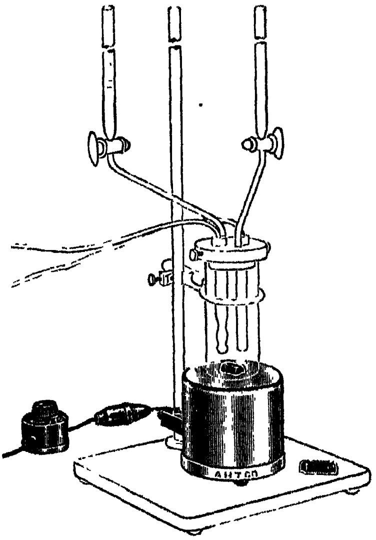

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INFRA-RED ABSORPTION SPECTRA OF TOCOPHEROLS AND RELATED STRUCTURES*

BY HARRIS ROSENKRANTZ

(*From the Russell Sage Institute of Pathology, The New York Hospital, and the Department of Physiology, Cornell University Medical College, New York City*)

(Received for publication, October 31, 1947)

The present report is based on an investigation of the infra-red absorption spectra of natural and synthetic vitamin E substances and structurally related compounds. Such spectra can be helpful through rapid identification of chemical products in the isolation and synthesis of tocopherols. It is also hoped that they may aid in the identification of the various tocopherols and oxidative products which might be isolated from the blood and urine of patients fed vitamin E extracts. This can be important, as it has recently been demonstrated that α-, γ-, and δ-tocopherols show quantitative differences in physiological activity in patients suffering from progressive muscular dystrophy.[1]

Methods

The infra-red spectra were obtained on the Hardy two beam spectrophotometer (1) and were recorded in the range from 2 to 12 μ. The instrument has been reported in detail by Furchgott *et al.* (2), but since then it has been designed to record automatically with a General Motors breaker type amplifier and a Brown potentiometer recorder. A cam control for the slits was also introduced in order to obtain a uniform intensity level over the region observed.

The samples were investigated as solid films on rock salt plates. Most of them were oils, smears being made between two NaCl plates; others were prepared from pyridine and carbon tetrachloride solution, the solvent being evaporated on a hot-plate. The oil smears gave better resolution and definition; however, there was a small shift in some bands when a crystalline film was used.

EXPERIMENTAL

The absorption spectra shown here are copies of original tracings from the automatic recorder, preserving band position and intensity but adjusting the base lines. The adjustment of base lines was necessitated

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* Aided by a grant from the Office of Naval Research under Contract N-6-ORI-91, Task Order 10.

[1] Milhorat, A. T., personal communication.

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because the cam control of the slit widths was not theoretically correct in giving a uniform intensity distribution. However, this did not affect the position of the absorption bands. The absorption curves of the following substances were obtained: natural α-, γ-, and δ-tocopherol, natural α-tocopherol acetate, succinate, and palmitate, natural γ-tocopherol palmitate, synthetic α-tocopherol and its acetate, α-tocopherylquinone, α-tocopherylhydroquinone triacetate, 2,5,6-trimethylhydroquinone, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, and 1,4-benzoquinone.[2]

The infra-red absorption spectra of synthetic *dl*-α-tocopherol and its acetate, although not shown in this paper, were found to be nearly identical with the spectra of natural *d*-α-tocopherol and its acetate respectively. The isolation of δ-tocopherol was reported by Weisler, Robeson, and Baxter[3] and we were fortunate in obtaining a small sample from them (3). There is a fourth vitamin E substance known as β-tocopherol which we have not studied at the present.

TABLE I

Absorption Maxima in Hydroxyl Region

Compound	Type O—H	Wave-length
		μ
α-Tocopherol	Phenolic	3.02
γ-Tocopherol	"	3.02
δ-Tocopherol	"	3.01
2,5,6-Trimethylhydroquinone	"	3.03
α-Tocopherylquinone	Alcoholic	2.93
α-Tocopherol succinate	Acid hydroxyl	3 ?

Analysis of Spectra

The infra-red absorption bands which can be assigned to specific atomic linkages will be discussed first.

O—H Absorption—Many of the compounds studied have one or more hydroxyl groups, whose linear vibrations appear in the 3 μ region of their spectra. Table I lists the wave-lengths of the absorption maxima observed in this region. It can be seen that the phenolic hydroxyl groups absorbed between 3.01 and 3.03 μ, while the alcoholic hydroxyl group absorbed about 2.93 μ. These values appear to be higher than the 2.75 to 2.80 μ absorption range for unbonded O—H groups. Furchgott *et al.* (4) have postulated intermolecular bonding for this shift to longer wave-lengths in estrogens, which also have a phenolic structure. The acid hydroxyl of α-tocopherol succinate gave a side bump in the alcoholic hydroxyl region. However, the major absorption from this acid hydroxyl was probably shifted into the C—H region because of the strong hydrogen bonding associated with acid groups (5).

---

[2] We wish to express our gratitude to Dr. Ade T. Milhorat of The New York Hospital, for obtaining many of these compounds from Distillation Products, Inc., for us.

[3] Meeting of the American Chemical Society, Chicago, September, 1946.

C—H Absorption—Other workers (6) have demonstrated that C—H groups of benzene rings absorb near 3.25 μ, while bands due to aliphatic type C—H linkages occur near 3.41 μ. In all these compounds studied here which have the long phytyl chain, the absorption maxima for the linear C—H linkage occurred at about 3.41 μ. In the case of the quinones which contain all or mainly aromatic C—H linkages, the absorption band occurred nearer 3.23 μ.

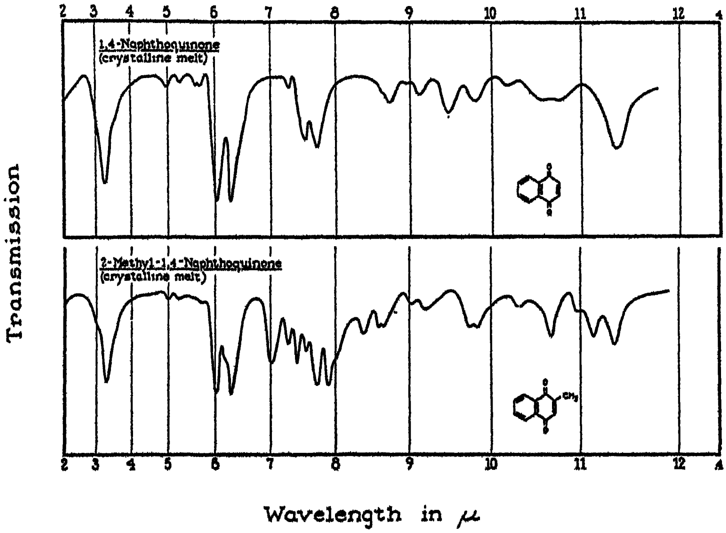

FIG. 1

Absorption bands also arise in the region near 7 μ from the angular vibration of C—H linkages (6). A series of bands is resolved between 6.7 and 7.15 μ which can be attributed to the aliphatic type C—H linkages (6). A clear cut example of this is shown through comparison of naphthoquinone and 2-methylnaphthoquinone (Fig. 1). In the latter compound, a band appeared at 7.02 μ which did not occur in the former. This is probably due to angular vibrations of hydrogen atoms of the methyl group attached to the aromatic nucleus. Such vibrations, in the case of methyl groups in saturated aliphatic molecules, appear to absorb at a somewhat lower wave-length (see the spectra presented by Barnes *et al.* (6)).

CH_3 Absorption—In the spectra of tocopherols, a band consistently occurred near 7.27 μ. On the basis of other studies (6) on the absorption of the CH_3 groups and our comparison between methylnaphthoquinone and non-methylated quinones, this band was attributed to the methyl groups present in these vitamin E compounds.

Naphthoquinone showed a band at 7.28 μ, but this is very weak compared with the bands near this wave-length found in the spectra of the tocopherols and of methylnaphthoquinone (Fig. 1).

C=O Absorption—The three types of carbonyl groups encountered in this study were ester, acid, and quinone. The ester type, as found in α-tocopherol acetate, succinate, palmitate, and γ-tocopherol palmitate. absorbed at about 5.70 μ (Fig. 2). In the α-tocopherol succinate we also find an acid type carbonyl which absorbed at 5.87 μ.

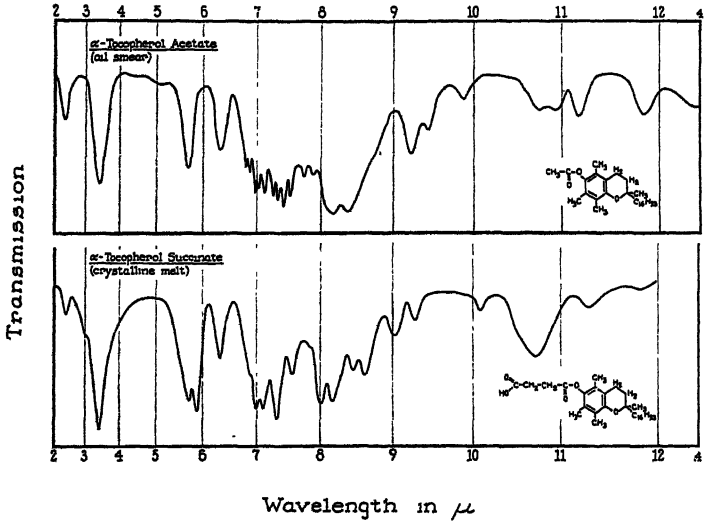

FIG. 2

The naphthoquinones and 1,4-benzoquinone were examined to help locate the absorption band arising from the quinone type carbonyl in α-tocopherylquinone. It can be seen from Fig. 3 that all the quinones gave a strong band at 6.03 μ. This band has been assigned to the quinone type C=O vibration.

C=C Absorption—The conjugated C=C system of the benzene and substituted benzene molecules has been previously shown (6) to give rise to a strong band in the 6.15 to 6.35 μ region. In the spectra of phenol (6) a strong band appears at about 6.27 μ. A strong band occurred consistently near 6.3 μ in these tocopherols and has been assigned to the phenolic type structure. Such a band also appeared in the quinones, but was more intense. This may be due to the more highly conjugated systems of the quinones.

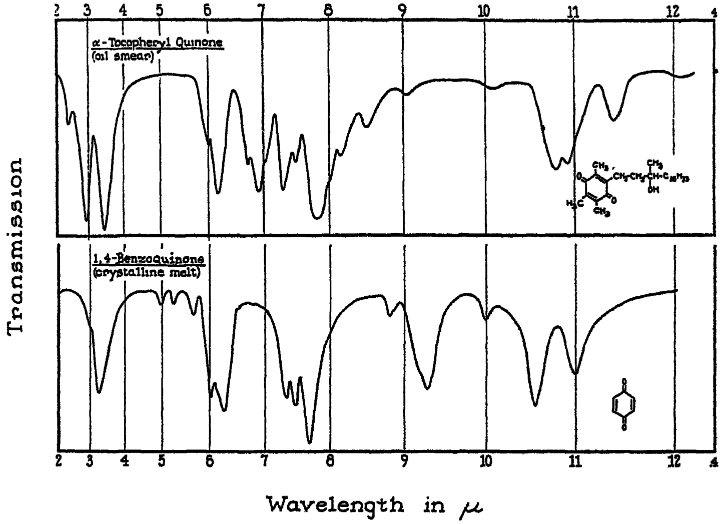

FIG. 3

TABLE II

Probable Absorption Maxima of Phenolic C—O Linkage

Compound	Wave-length
	μ
α-Tocopherol	7.85
γ-Tocopherol	8.17
δ-Tocopherol	8.28
α-Tocopherol acetate	8.18
α-Tocopherol palmitate	8.06
α-Tocopherol succinate	8.16
γ-Tocopherol palmitate	8.14
α-Tocopherylhydroquinone triacetate	8.08
2,5,6-Trimethylhydroquinone	?*

* See the text.

Phenolic C—O Absorption—It appears probable that the intense absorption band near 8 μ in the tocopherols results from the C—O vibration of the phenolic hydroxyl. Previous work (4, 6) has indicated that C—O linkages, where the C has one double bond, give rise to bands in this region. The influence of methylation and esterification was observed on the 8 μ band.

Substitution of methyl groups on the tocopherol molecule resulted in a shift of the 8 μ band to a shorter wave-length. Table II shows this shift as one goes from the monomethyl-δ-tocopherol to the trimethyl-α-tocopherol. This band occurred at 8.28 μ in δ-tocopherol, 8.17 μ in γ-tocopherol, and at 7.85 μ in α-tocopherol. Esterification of α-tocopherol appeared to shift the 8 μ band to a longer wave-length (Table II). The 7.85 μ band of α-tocopherol was shifted to 8.18 in the acetate, 8.06 in palmitate, and 8.16 μ in the succinate.

The trimethylhydroquinone which also has the phenolic type C—O linkage gave a split band near 7.6 μ and another band at 8.23 μ. It is not known at this time which of these bands resulted from the phenolic hydroxyl C—O vibration (Fig. 4).

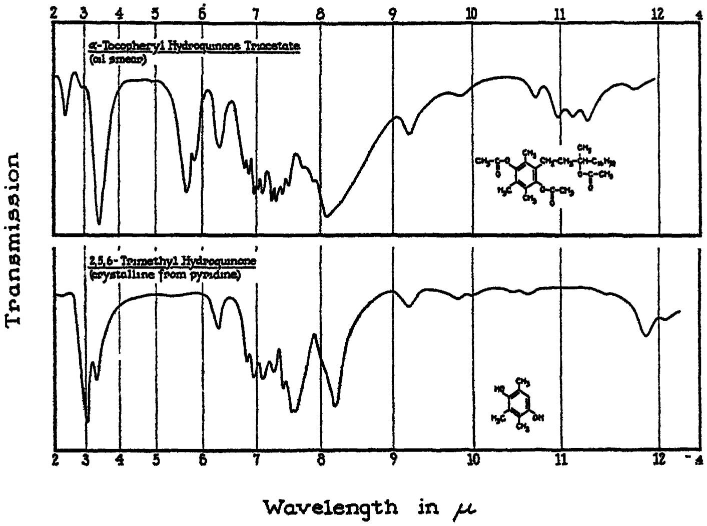

FIG. 4

Other Absorption Bands—In the spectra of this group of tocopherols, there are other absorption bands which cannot as yet be assigned to specific interatomic vibrations. Many of these "unassigned" bands occurred between 10 and 12 μ. Absorption bands occurring near 10 μ probably arise from C—C vibrations (6). Many of the others undoubtedly result from complex vibrations involving the chroman nucleus itself as well as the long phytyl chain. With this possibility in view, cross-comparisons were made of all the absorption spectra obtained in this study to see whether any relationship existed.

The spectra of the tocopherols studied here showed a band near 8.6 μ, This band was probably masked in α-tocopherol acetate by the broad, intense bands near this region. However, its medium intensity and its consistency indicated that it might be a characteristic band of the tocopherol molecule. In this respect, it is interesting to observe the marked increase in intensity of this 8.6 μ band in the α- and γ-tocopherol palmitates (Fig. 5). This increase in intensity is probably due to an effect by the long fatty acid side chain. Examination of spectra published by Barnes *et al.* (6) of esters of long chain fatty acids demonstrated the presence of a strong band near this wave-length.

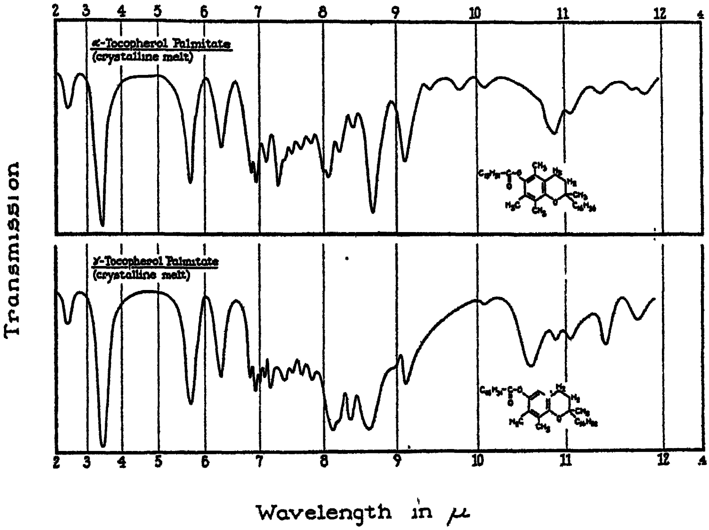

FIG. 5

The region between 10.6 and 11.0 μ in the spectra of the tocopherols and their derivatives contained one or two strong bands. The variations of wave-lengths of these bands in the free tocopherols make them useful for differentiation. They are probably characteristic bands of the tocopherol structure.

There seemed to be some consistency among the compounds as to absorption bands which occurred near 11.4 and 11.8 μ. However, the large variation in intensity of these bands offered too much difficulty in interpretation.

An absorption band occurred in the tocopherols and the two oxidative products of α-tocopherol near 2.4 μ. This wave-length did not correspond to the atmospheric water vapor bands in this region. Several estrogens which contain a phenolic configuration in Ring A were studied, and no bands near 2.4 μ were found. We are not certain whether this band is an artifact or an overtone.

In conclusion, it may be pointed out that the various tocopherols, as well as their derivatives, may be easily distinguished by their infra-red absorption spectra (Fig. 6). The tocopherol structure itself appears to be characterized by certain absorption bands. These bands include several assigned absorption bands resulting from vibrations of the phenolic hydroxyl, the benzene ring, and the phenolic C—O linkage, as well as certain "unassigned" absorption bands occurring near 8.6 and 10.9 μ.

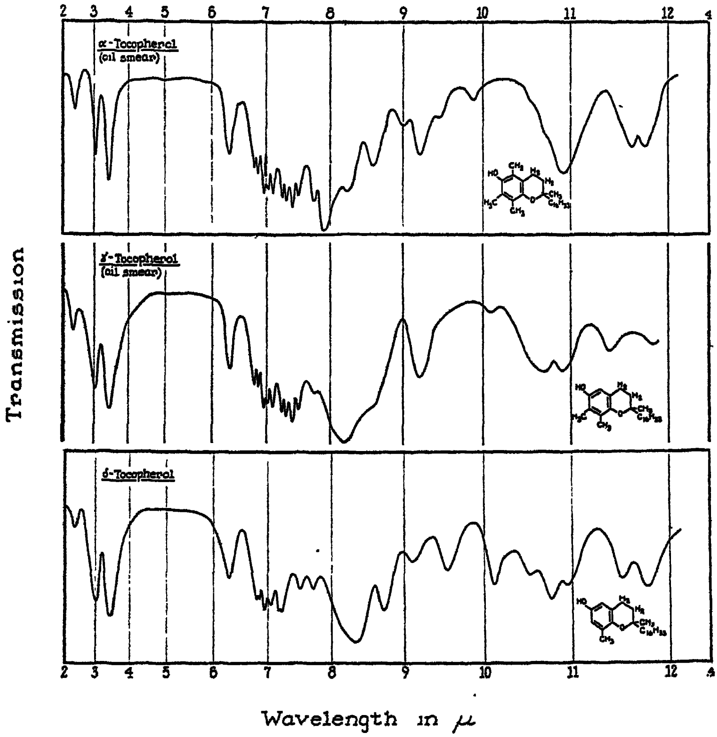

FIG. 6

SUMMARY

1. The infra-red absorption spectra, from 2 to 12 μ, of nine free and substituted tocopherols and six related structures have been presented.

2. Certain absorption bands in the spectra have been discussed in relation to the chemical structure of tocopherols.

3. Assigned absorption bands near 3.0, 6.3, and 8.0 μ in addition to

unassigned bands near 8.6 and 10.9 μ are characteristic of the tocopherol structure.

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APPLICATION OF AN IMPROVED GLUCURONIDASE ASSAY METHOD TO THE STUDY OF HUMAN BLOOD β-GLUCURONIDASE*

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Previous conditions in which phenolphthalein glucuronide was employed as substrate (1) have proved unsatisfactory for the determination of glucuronidase in plasma, serum, and laked blood cells. Thus, low glucuronidase activities such as frequently occur in plasma could not be accurately measured or even approximated, owing to a combination of circumstances; *i.e.*, too short an incubation period often coupled with interference by a turbid state of the plasma and the presence in it of biliary and carotenoid pigments. Moreover, solutions of laked erythrocytes could not be assayed because of the red color of the hemoglobin. These difficulties have now been largely overcome by the introduction of a deproteinizing procedure and by otherwise modifying the conditions of assay. It has been possible also to simplify greatly the biosynthetic process of manufacturing the substrate, phenolphthalein glucuronide, which is now in demand.[1]

The interpretation of blood glucuronidase values is handicapped by our lack of knowledge concerning the factors which control the level of this enzyme in the blood; *e.g.*, we do not know to what extent the plasma glucuronidase is influenced by hormonal factors. The nature and significance of the distribution of β-glucuronidase between the plasma and the formed elements of the blood is likewise not understood. As a result of observations made on blood glucuronidase with the improved assay technique, some of the desired information has been obtained. An account of the method and its use in these experiments is given in the present paper.

Method

Simplified Preparation of Substrate, Phenolphthalein Glucuronide—The filtered, toluene-free urine obtained from rabbits receiving injections of sodium phenolphthalein phosphate[2] was acidified to Congo red paper with

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[1] Private communications.

[2] This product is now made more conveniently than before (2). First, dry chloroform (40 cc.) and then a mixture of (redistilled) phosphorus oxychloride (50 cc.) and chloroform (50 cc.) was added with mechanical stirring to 50 gm. of phenolphthalein in

6 N hydrochloric acid with vigorous shaking, and 800 cc. portions of urine were extracted with four successive 125 cc. portions of ethyl acetate. The ethyl acetate phase was centrifuged, dried by decantation through cotton, and reduced to small volume *in vacuo* at 50°. This was added to an excess of saturated cinchonidine in ethyl acetate. The cinchonidine derivative of phenolphthalein glucuronide so obtained was crystallized by dissolving it in the minimum quantity of hot methyl alcohol and adding 4 volumes of hot ethyl acetate. One more such crystallization was required in order to obtain a product which, according to analyses for hydrolyzable phenophthalein, is pure phenolphthalein mono-β-glucuronide with 1 molecule of methyl alcohol of crystallization. The yield of product has been greatly improved by this process.

Method for Blood Glucuronidase—Plasma and formed elements of the oxalated or heparinized blood (14 cc. divided between two Wassermann tubes) were separated by centrifugation. The buffy coat was aspirated with a capillary pipette, after removal of the plasma, and was laked with 10 cc. of distilled water after alternate freezing (in a carbon dioxide snow-acetone mixture) and thawing at room temperatue, to facilitate lysis. The red cells were laked with three volumes of distilled water and the total volume was recorded. The plasma was usually filtered through a Seitz filter before sampling it for assay. Blood serum was sampled without any preliminary treatment.

Into two Wassermann tubes were pipetted 0.1 cc. of glucuronidase-containing solution, 0.8 cc. of 0.1 M acetate buffer (1), pH 4.5, and 0.1 cc. of 0.01 M phenolphthalein glucuronide (1). A third Wassermann tube containing the enzyme and buffer but not the substrate served as the control. The digests were then incubated at 38° and the time recorded. For blood plasma, serum, and cells, an incubation period of between 15 and 24 hours was required in contrast to a 1 to 5 hour period for most tissues.

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a 1 liter round bottom flask cooled in an ice bath. To this was added dropwise dry pyridine (40 cc.), with stirring continued for a total of 3 to 5 hours. The next day, 150 cc. of distilled water were added in small quantities to the ice-cooled reaction flask, followed by an excess of 40 per cent sodium hydroxide (approximately 300 cc.). Upon cooling, needle-like crystals (sodium phosphate) appeared and were removed by filtration. When an excess of concentrated hydrochloric acid (Congo red paper) was added to the aqueous phase, phenolphthalein diphosphoric acid precipitated as a gum which could be conveniently separated from the mixture with a glass stirring rod. This gum was warmed in a porcelain dish on a boiling water bath and 20 cc. of concentrated sodium hydroxide (100 gm. plus 100 cc. of distilled water) were added gradually with stirring. Solution of the gum was completed with distilled water (final volume 200 cc.). 3 cc. of this phenolphthalein phosphate were brought to neutrality with weak alkali and diluted to 10 cc. with distilled water. This amount was injected subcutaneously daily at two widely separated sites on the skin of each rabbit for 6 days. The animals received carrots and cabbage, water being withheld. The urine collections under toluene were completed on the morning of the 8th day.

At the end of the incubation period, 1.0 cc. of 5 per cent trichloroacetic acid was added to each tube with thorough mixing. To digests prepared from laked cells was added 1.0 cc. of 10 per cent trichloroacetic acid. This step served to stop the reaction and to deproteinize the digest. The time was recorded. The tubes were centrifuged at high speed for 10 minutes. The contents were decanted through cotton plugs held in the stem of small glass funnels into colorimeter tubes marked at 6 cc. The colorimeter tubes contained 2.5 cc. of an alkaline reagent mixture (200 cc. of glycine buffer of pH 10.45 (1) plus (*a*) 50 cc. of 0.5 N sodium hydroxide in the case of digests deproteinized with 5 per cent trichloroacetic acid or (*b*) 50 cc. of 1.0 N alkali when 10 per cent trichloroacetic acid was used). The precipitate remaining after decanting was suspended in 1.0 cc. of distilled water, centrifuged, and the supernatant decanted through the same filter. Washing of the tube and funnel was continued until the filtrate amounted to 6 cc. 0.1 cc. of 0.01 M phenolphthalein glucuronide was added to the control tube, which was used in the colorimeter to obtain the setting at 100. After thorough mixing, readings were made with the 6.0 cc. well and the 540 mμ filter. The L (optical density) values were substituted in a phenolphthalein calibration curve (1) previously prepared with the present volumes of the various constituents of the enzyme digest.

When fresh reagents are employed, it is important to check the pH of the final alkalinized mixture (pH 10.2 to 10.4) or the color development of a known amount of phenolphthalein.

Calculations

Serum, Plasma, or Body Fluids

$$\text{Micrograms phenolphthalein liberated in digest} \times \frac{1}{\text{hrs. of incubation}}$$

$$\times \frac{100}{\text{cc. fluid analyzed}} = \text{units glucuronidase per 100 cc. fluid}$$

Laked Blood Cells[3]

$$\text{Micrograms phenolphthalein liberated in digest} \times \frac{1}{\text{hrs. of incubation}}$$

$$\times \frac{\text{volume laked cells}}{0.1} \times \frac{100}{\text{volume original blood specimen}}$$

$$= \text{units glucuronidase per 100 cc. whole blood}$$

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[3] It has been considered best for the present to relate the activity of laked blood cells to a 100 cc. volume of whole blood. The buffy coat, especially, consists of a heterogeneous mixture of cell types and so the use of units based on the number of cells only would be meaningless in the opinion of a histologist. However, it should be pointed out that the values for buffy coat glucuronidase do not properly indicate the great magnitude of the glucuronidase activity which must exist in the white cells as compared to an equivalent number of tissue cells.

Tissues

$$\text{Micrograms phenolphthalein liberated in digest} \times \frac{1}{\text{hrs. of incubation}}$$

$$\times \frac{\text{volume extract}}{0.1} \times \frac{1}{\text{gm. tissue}} = \text{units glucuronidase per gm. tissue}$$

The changes which have been introduced in the method have in no way altered the principle upon which the test was based originally; *i.e.*, the colorimetric determination of phenolphthalein liberated by enzymatic hydrolysis of phenolphthalein glucuronide. Optimal conditions of hydrolysis have been maintained and very good agreement in duplicate analyses was obtained as before (1). The present procedure has greater flexibility in its application and is not affected by the physical state or the pigment content of the specimen. Great economy of substrate is achieved without sacrificing any of the desirable features of the original method. A few data on the blood and tissues of cancer patients have already appeared (3).

TABLE I

Distribution of β-Glucuronidase in Blood

Healthy adults	Glucuronidase activity			Morphology of white blood cells			
	Plasma, units per 100 cc.	Red cells, units per 100 cc. whole blood	Buffy coat, units per 100 cc. whole blood	No. per c.mm	Polymorphonuclear leucocytes	Lymphocytes	Other cells
					per cent	*per cent*	*per cent*
C. ♂	37	0	818	7,800	27	73	0
S. ♂	84	5	378	5,500	61	37	2
G. ♂	140	0	307	5,400	33	60	7
P. ♂	121	0	614	9,500	37	62	1
M. ♂	0	10	780	21,400	68	30	2
L. ♂	37	0	384	8,500	73	27	0
H. ♂	0	0	353	12,700	58	42	0
J. ♂	150	0	261	8,200	66	30	4
L. ♂	181	18	425	9,500	60	40	0
H. ♂	79	7	655	10,200	67	33	0
S. ♂	118	3	647	7,400	86	14	0
G. ♀	96	0	318	4,800			
B. ♀	84	0	426	7,900	47	47	6
S. ♀	179	0	528	7,000	57	40	3
W. ♀	230	0	423	13,500	90	10	0
G. ♀	86	8	342	8,200	45	50	5

EXPERIMENTAL

The distribution of glucuronidase activity between plasma, erythrocytes, and the buffy coat was determined in sixteen subjects with the method described. These values are compared with white cell morphology in Table I.

Platelet-rich plasma was prepared by slow centrifugation of 14 cc. of heparinized blood. After removal of this platelet-rich plasma, the blood cells were centrifuged at high speed and the platelet-poor buffy coat aspirated and laked with 10 cc. of water after an intermittent freezing-thawing procedure. Glucuronidase determinations were done on the plasma before and after Seitz filtration and on the laked buffy coat. These values have been correlated with plasma platelet counts in Table II.

TABLE II

Study of Platelet Glucuronidase Activity

Subject	No. of platelets in plasma per c.mm.	Platelet-rich plasma, units per 100 cc.	Platelet-free plasma, units per 100 cc.	Platelet-poor buffy coat, units per 100 cc. whole blood
S.	140,000	166	103	759
Y.	170,000	146	118	854
B.	300,000	76	73	580
L.	440,000	58	37	874
S.	400,000	164	171	200
W.	500,000	59	72	333

TABLE III

Effect of Repeated Washing with Tyrode's Solution on Buffy Coat Glucuronidase

	Subject 1*		Subject 2*	
	Activity, units per 100 cc. blood	Per cent total buffy coat glucuronidase	Activity, units per 100 cc. blood	Per cent total buffy coat glucuronidase
1st washing	125	7.3	111	8.9
2nd "	51	3.0	104	8.3
3rd "	28	1.6	60	4.8
4th "	68	4.0	98	7.8
5th "	14	0.8	21	1.7
6th "	44	2.6	71	5.6
7th "	64	3.8	29	2.3
Water-laked washed buffy coat	200	11.8	292	23.2
Freezing-laked residue after water-laking	1110	65.1	472	37.4
Total buffy coat glucuronidase	1704		1258	

* White cell count of Subject 1, 8400 per c.mm.; of Subject 2, 10,800

In Table III, two cases are illustrated of the effect of repeated washing with Tyrode's solution on buffy coat glucuronidase. The buffy coat of

14 cc. of blood was separated and evenly suspended with gentle stirring in 10 cc. of Tyrode's solution. The mixture was centrifuged and the supernatant removed for glucuronidase assay (first washing). After seven such washings, the buffy coat was laked with distilled water (10 cc.), centrifuged, and the supernatant (water-laked buffy coat) assayed. The residue was subjected to alternate freezing and thawing at room temperature. The material was suspended in 10 cc. of H_2O, centrifuged, and the supernatant assayed (freezing-laked residue). The white cells remained intact during washing with Tyrode's solution. However, suspending them in distilled water afterwards accomplished almost complete laking.

Results

From Table I it is clear that the red cells contain little or no glucuronidase and that the buffy coat contains the major portion of the blood glucuronidase activity. Similar findings have been made with the blood of the mouse, rat, rabbit, and dog. There does not seem to be any correlation of the buffy coat glucuronidase with the percentage of polymorphonuclear leucocytes and lymphocytes in the blood. Although greater glucuronidase activities are frequently found in the presence of high white cell counts, a strict dependence of the glucuronidase level upon the number of white blood cells is not evident. This may be due in part to variations in the amount of buffy coat which can be recovered for assay.

The platelet-rich plasma contains a small number of white cells which are rich in glucuronidase. The removal of these cells rather than the platelets probably explains the small decrement in activity which follows Seitz filtration (Table II). It may be concluded that the platelets contain little or no glucuronidase acitvity.

A substantial fraction of the total buffy coat glucuronidase can be removed by repeatedly suspending the cells in Tyrode's solution (Table III). The disruption of the cell membranes by water laking did not release as much enzymic activity as appeared in the lysate after the intermittent freezing-thawing procedure.

DISCUSSION

The presence of β-glucuronidase activity in blood was first observed and studied by Fishman with a view to demonstrating a possible correlation with the process of the "metabolic conjugation" of the estrogenic hormones. While the blood of castrated female mice showed no change in glucuronidase activity following estrogen injection (4), the blood glucuronidase of women underwent characteristic alterations correlated with the events of pregnancy (3).

It was then clearly established that the greater part of the blood glucuronidase resided in the formed elements composed of erythrocytes, platelets, leucocytes, and lymphocytes. The present data strongly indicate that the erythrocytes and platelets may be eliminated as important sources of blood cell glucuronidase. In view of the relatively low glucuronidase activity of normal lymph node tissue (5, 6), one would expect that the lymphocytes should possess less of this enzyme that the leucocytes. On the other hand, the lack of correlation of buffy coat glucuronidase with the relative proportion of lymphocytes and leucocytes would not be in favor of this prediction. For the time being, final conclusions should not be drawn until other experiments are done in which the influence of blood factors affecting glucuronidase[4] have been properly taken into account.

The liberation of β-glucuronidase activity from the buffy coat of the blood by washing with Tyrode's solution is of interest with regard to the question of the distribution of β-glucuronidase in the living cell. In view of the low content of glucuronidase in erythrocytes and in platelets, it seems unlikely that the enzyme is merely adsorbed by the cell membrane from the plasma in a non-specific manner. However, the possibility does exist that the enzyme is a cytoplasmic constituent, capable of being secreted through the cell membrane, since β-glucuronidase does occur in secretions of the glandular epithelium (saliva, gastric juice, urine, spinal fluid, tears, bronchial secretions), the range of glucuronidase activity being 50 to 800 units per 100 cc.

In view of the known secretory functions of malignant neoplastic cells, one may expect to find glucuronidase in both the cells and stroma of cancer tissue.

Under the conditions which obtain *in vivo*, one wonders to what extent plasma or serum glucuronidase may be derived from the white blood cells, either through their breakdown or possibly through secretion. In this regard, it seems likely that the white cells ordinarily contribute little glucuronidase to the plasma, since many normal individuals have practically no plasma glucuronidase activity in the presence of high buffy coat enzymic activity. However, this point should be kept in mind in the study of blood glucuronidase in disease.

The explanation of the function of the enzyme in the white blood cells is not apparent at present. Previously, it has been suggested that β-glucuronidase functions in processes of "metabolic conjugation", with much attention being directed towards the formation of steroid glucuronides. However, another possibility also exists; *e.g.*, other glucuronides of greater molecular weight, such as mucin, hyaluronic acid, chondroitin sulfate, and heparin may be synthesized in part through glucuronidase activity. Such syntheses may or may not be related to estrogen metabolism. In this regard it should be pointed out that intercellular mucin can be increased by the administration of estrogenic hormones (7–11).

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[4] Fishman, W. H., Altman, K. I., and Springer, B., *Federation Proc.*, in press.

SUMMARY

The method for determining glucuronidase activity with phenolphthalein glucuronide as substrate has been adapted to the assay of blood glucuronidase. This procedure has the advantages of greater flexibility in its use and of freedom from interfering substances in blood. In addition, the process of manufacturing biosynthetic phenolphthalein glucuronide has been improved and simplified.

By means of this method, it has been possible to show that the major portion of the glucuronidase activity of the blood is concentrated in the leucocytes and lymphocytes of the formed elements, little or none being present in erythrocytes and platelets. A substantial amount of white blood cell glucuronidase can be removed from the intact cells by washing them with Tyrode's solution. β-Glucuronidase activity has been demonstrated in saliva, gastric juice, spinal fluid, urine, and tears, which suggests that the enzyme can be secreted by the glandular epithelium. These observations have been discussed in relation to the possible function of the enzyme in processes of metabolic conjugation.

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ON THE PROTEOLYTIC ENZYMES OF ANIMAL TISSUES

VII. A PEPTIDASE OF CALF THYMUS*

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It was noted in an earlier paper of this series (1) that the sera of several animals and the extracts of various tissues (skin, lung, intestinal mucosa, etc.) contain a peptidase which rapidly hydrolyzes L-leucylglycylglycine (L-LGG) to leucine and glycylglycine. This peptidase is different from the previously recognized leucine aminopeptidase, since it does not hydrolyze L-leucinamide (L-LA) and is not activated by manganese ions. Its widespread distribution in tissues has raised the possibility that the tripeptide-splitting enzyme is not derived from the characteristic cells of a particular tissue such as skin or lung, but from cells such as lymphocytes which may be present in that tissue. Furthermore, the occurrence of this peptidase in serum has been attributed to its liberation into the circulating body fluids in the course of the rapid turnover of lymphoid cells (1). This view has received support from the finding that the administration to mice of adrenal cortical extracts or of pituitary adrenotropic hormone results in the elevation of the serum peptidase activity toward L-LGG (2). In the light of the prior demonstration that the rate of turnover of lymphoid tissue is under pituitary-adrenal control (3), this result encourages the conclusion that a significant portion of the serum peptidase activity is derived from the disintegration of lymphoid cells. These developments have prompted the study of the proteolytic activity of extracts of tissues known to be rich with respect to lymphocytes. In the present communication, data are presented concerning some of the proteolytic enzymes found in extracts of calf thymus.

Proteolytic Activity of Crude Saline Extracts of Calf Thymus—When calf thymus is extracted with 2 per cent sodium chloride solution, the resulting solution exhibits considerable proteolytic activity toward tripeptides such as L-LGG and diglycylglycine (GGG). As will be noted in Table I, following the addition of manganese ions, the hydrolysis of L-LGG is accelerated and L-LA is split rapidly, thus indicating the presence, in the saline extract, of a manganese-activatable leucine aminopeptidase. The typical substrate for trypsinases, benzoyl-L-argininamide, and a substrate for pepsinases,

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carbobenzoxy-L-glutamyl-L-tyrosine, are not hydrolyzed appreciably, and similar negative results were obtained with carbobenzoxyglycyl-L-phenylalanine, the typical substrate for carboxypeptidases. The presence of a manganese-activatable prolidase (substrate, glycyl-L-proline) could be demonstrated, however. It would appear, therefore, that, in addition to leucine aminopeptidase and prolidase, the saline extract of calf thymus contains appreciable amounts of a tripeptide-splitting peptidase similar to that noted previously in extracts of skin and lung and in serum. As will be shown in this communication, this enzyme hydrolyzes both L-LGG and GGG, and in what follows it will be referred to as lymphopeptidase.[1]

TABLE I

Proteolytic Activity of Saline Extract of Calf Thymus

Enzyme concentration, 0.28 mg. of protein nitrogen per cc. of test solution.

Substrate	pH	Time	Hydrolysis		
			No activator added	0.001 M $MnSO_4$ present	0.01 M cysteine present
		hrs.	*per cent*	*per cent*	*per cent*
L-Leucylglycylglycine	7.8	1	32	72	35
		2	60	103	56
Diglycylglycine	7.8	1	20	12	19
		2	39	25	37
L-Leucinamide	7.7	2	6	57	4
		4	14	94	8
Benzoyl-L-argininamide	5.0	7			3
	7.6	7	2		
Carbobenzoxy-L-glutamyl-L-tyrosine	5.4	6	1		
Carbobenzoxyglycyl-L-phenylalanine	5.1	6			2
	7.6	6	0		
Glycyl-L-proline	8.0	4	5	21	

Partial Purification of Lymphopeptidase—Appreciable concentration of lymphopeptidase activity may be effected by heating the crude saline extract to 50°, followed by fractional precipitation between 0.4 and 0.7 saturation with respect to ammonium sulfate. Prolonged dialysis against distilled water gives enzyme solutions which are notably enriched in lymphopeptidase and which show little or no leucine aminopeptidase or prolidase activity (Table II). This enzyme preparation has been used in the experiments reported in the succeeding sections of this paper.

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[1] Since the lymphoid cells of calf thymus constitute only about 60 per cent of the cellular mass, exception may perhaps be taken to the designation of the tripeptide-splitting enzyme as a constituent of lymphocytes. Support for the use of the term "lymphopeptidase" is provided by unpublished studies on the mesenteric lymph node of the mouse. This tissue, which is characterized by an overwhelming predominance of lymphocytes, also exhibits extremely high proteolytic activity toward L-LGG and GGG.

As in the case of other tissue extracts (1), such solutions of lymphopeptidase, under our experimental conditions, hydrolyze L-LGG and GGG with the kinetics of a zero order reaction (*cf.* Table III), and the rate may therefore be defined by a constant K^0 which equals per cent hydrolysis per minute. In Table III, the proteolytic coefficient C is defined as K/(enzyme concentration (expressed as mg. of protein nitrogen per cc. of test solution)).

TABLE II

Proteolytic Activity of Partially Purified Lymphopeptidase

Enzyme concentration, 0.009 mg. of protein nitrogen per cc. of test solution; pH 7.8 to 8.0.

Substrate	Time	Hydrolysis	
		No activator added	0.001M $MnSO_4$ present
	hrs.	*per cent*	*per cent*
L-Leucylglycylglycine	1	25	26
	2	49	53
	4	91	90
	6	98	99
Diglycylglycine	1	23	21
	2	44	40
	4	81	
	8	97	
L-Alanylglycylglycine	1	24	24
	2	47	46
	4	86	
	8	99	
L-Leucinamide	6	1	2
Glycyl-L-proline	2	0	1
	6	2	7

Calculation of the proteolytic coefficients of the crude saline extract of thymus and of the partially purified lymphopeptidase solution shows a 33-fold increase in C_{GGG} (from 1.2 to 40). The increase in C_{LGG} is somewhat less (25-fold), which may be attributed to the presence in the crude extract of some active leucine aminopeptidase which is eliminated in the course of the purification.

Preliminary experiments have shown that it is possible to concentrate lymphopeptidase further by adsorption at pH 6 on Cγ alumina, followed by elution with M/15 phosphate buffer at pH 7.4. Fractional precipitation

TABLE III

Kinetics of Hydrolysis of L-*Leucylglycylglycine and of Diglycylglycine by Lymphopeptidase*

The pH was maintained at 7.9 in all cases.

Enzyme concentration, protein N per cc. test solution	Time	L-Leucylglycylglycine			Diglycylglycine		
		Hydrolysis	K^0_{LGG}	C^0_{LGG}*	Hydrolysis	K^0_{GGG}	C^0_{GGG}*
mg.	*min.*	*per cent*			*per cent*		
0.0045	60	12	0.20	47	10	0.17	39
	90	20	0.22		15	0.17	
	120	25	0.21		21	0.18	
	180	38	0.21		32	0.18	
0.009	60	25	0.42	46	22	0.37	41
	90	37	0.41		34	0.38	
	120	49	0.41		44	0.37	
0.0135	30	19	0.63	47	17	0.57	40
	60	38	0.63		31	0.52	
	90	58	0.64		47	0.52	
0.018	30	26	0.87	48	23	0.77	43
	60	51	0.85		46	0.77	
0.027	30	38	1.27	47	32	1.07	39
	60	75	1.25		63	1.05	

* C^0 = The average of K^0 per mg. of protein nitrogen.

TABLE IV

pH Dependence of Lymphopeptidase Action

Enzyme concentration, 0.10 mg. of protein nitrogen per cc. of test solution.

pH	K^0	
	L-Leucylglycylglycine	Diglycylglycine
6.8	0.35	0.28
7.3	0.38	
7.5		0.34
7.6	0.41	
7.8	0.43	0.38
8.0	0.45	0.39
8.1		0.37
8.2	0.42	
8.4	0.40	0.32
8.7	0.35	

with ammonium sulfate has given enzyme solutions with proteolytic coefficients (C_{GGG}) of 85 to 102.

pH Dependence of Lymphopeptidase Action—The pH optimum for the action of lymphopeptidase on L-LGG or GGG is near pH 8 (*cf.* Table IV).

Specificity of Lymphopeptidase—As will be noted from Table II, partially purified lymphopeptidase hydrolyzes not only L-LGG and GGG but also L-alanylglycylglycine (L-AGG), and the rates of hydrolysis of these tripeptides are quite similar. It was reported previously (1) that the LGG-splitting enzyme in extracts of skin and in serum is readily inactivated at pH values below 5. A similar behavior is exhibited by lymphopeptidase, as is shown in Table V. In addition, following partial inactivation of the enzyme, the activity toward L-LGG, GGG, and L-AGG decreases in a parallel manner, thus supporting the view that the hydrolysis of the three tripeptides is effected by the same enzyme. It would appear, therefore, that the specificity requirements of lymphopeptidase permit of some variation in the nature of the amino acid residue which bears the free amino group.

The extent of hydrolysis of the tripeptides by lymphopeptidase does not exceed that expected for the hydrolysis of one peptide bond (*cf.* Table II). It has been shown previously (4, 1) that partially purified extracts of intestinal mucosa and of skin hydrolyze L-LGG at the peptide linkage adjacent to the free amino group. Lymphopeptidase also acts at this peptide linkage in L-LGG and hydrolyzes L-AGG at the analogous bond between the alanine and middle glycine residues. This is readily demonstrated by polarimetric observation of the course of hydrolysis, since the final rotation corresponds closely to the value for the mixture of the appropriate optically active amino acid and glycylglycine. In the case of L-LGG (0.05 M), after 100 per cent hydrolysis of one peptide linkage at pH 7.8, the reading was $\alpha_D = -0.04°$ (1 dm. tube, 25°), while a mixture of L-leucine (0.05 M), glycylglycine (0.05 M), enzyme (0.008 mg. of protein nitrogen per cc.), and veronal buffer gave a rotation of −0.05°. On the other hand, a comparable mixture of L-leucylglycine (0.05 M), glycine (0.05 M), enzyme, and buffer

TABLE V

pH Stability of Lymphopeptidase

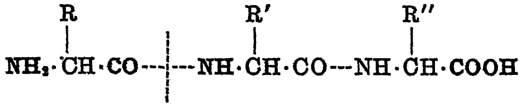
The pH of the enzyme solution was adjusted by the addition of suitable amounts of 0.1 N hydrochloric acid to the veronal buffer-enzyme mixture. After 1 hour at 40°, the pH was readjusted to pH 7.8 and the appropriate substrate was added. Enzyme concentration, 0.009 mg. of protein nitrogen per cc. of test solution.

pH of enzyme solution	K^0		
	L-Leucylglycylglycine	Diglycylglycine	L-Alanylglycylglycine
7.4	0.40	0.36	0.39
6.3	0.35	0.29	0.32
5.4	0.15	0.13	0.13
4.7	0.03	0.01	0.02
2.6	0.00	0.00	0.00

gave a reading of +0.55°. Similarly, for 100 per cent hydrolysis of one peptide linkage of L-AGG (0.05 M) at pH 7.8, the observed rotation was +0.01°, and the same value was obtained for a mixture of L-alanine, glycylglycine, enzyme, and buffer. A mixture of L-alanylglycine, glycine, enzyme, and buffer gave a reading of +0.21°. It may be concluded, therefore, that lymphopeptidase hydrolyzes L-LGG and L-AGG at the peptide linkage adjacent to the free amino group, and presumably splits the analogous peptide bond of other tripeptides which serve as substrates.

The data in Table VI show that the substitution of the free amino group of a substrate of lymphopeptidase, as in carbobenzoxy-L-LGG or carbobenzoxy-GGG, completely abolishes enzyme action. Since the action of lymphopeptidase is to hydrolyze LGG at the peptide bond between the leucine and middle glycine residues, this result suggests that the enzyme belongs to the group of aminoexopeptidases (5). Dipeptides such as glycylglycine, L-leucylglycine, or glycyl-L-leucine are not hydrolyzed to an appreciable extent, and it would appear that the presence of a free carboxyl group in adjacence to the sensitive peptide bond exerts an inhibitory effect on the enzymatic action.

The specificity of lymphopeptidase differs from that of previously characterized exopeptidases (aminopeptidase, carboxypeptidase), since it involves structural requirements in addition to a terminal free amino group and the peptide bond which is hydrolyzed. This is shown by the fact that glycylglycinamide and diglycylglycinamide are resistant to enzyme action, thus indicating the need for the presence, in a tripeptide, of an amino acid bearing a free α-carboxyl group. Of especial interest in this connection is the failure of lymphopeptidase to hydrolyze, at an appreciable rate, such tetrapeptides as triglycylglycine, diglycyl-L-leucylglycine, or diglycyl-L-glutamylglycine. It may be concluded, therefore, that lymphopeptidase is an enzyme which acts as an "aminoexotripeptidase" and which requires in its substrates the groups indicated in the following formula by means of bold-faced letters.



$$NH_2\cdot\overset{\substack{R\\|}}{CH}\cdot CO\text{---}\vdots\text{---}NH\cdot\overset{\substack{R'\\|}}{CH}\cdot CO\text{---}NH\cdot\overset{\substack{R''\\|}}{CH}\cdot COOH$$

It has been shown previously (6) that L-alanylsarcosylglycine is resistant to the action of the peptidase from intestinal mucosa which hydrolyzes L-AGG. A similar result was noted with lymphopeptidase (*cf.* Table VI), and this indicates the need for the "peptide hydrogen" in the peptide bond which is split by the enzyme. The requirement for the presence of a hydrogen atom in the sensitive peptide bond may be interpreted as evidence for the view, expressed earlier (7), that, in the case of several proteolytic enzymes, the peptide bond which is hydrolyzed undergoes enolization in the course of the enzymatic action. An exception to this conclusion is the highly specific prolidase which hydrolyzes peptide bonds involving the imino group of proline or hydroxyproline (6, 4).

If GGG is modified so as to insert a methyl group in place of the peptide hydrogen of the peptide linkage adjacent to the free carboxyl group, the resulting glycylglycylsarcosine is readily hydrolyzed by lymphopeptidase. Similarly, glycylglycyl-L-proline also is split by the enzyme. It would appear, therefore, that enolization of the peptide bond adjacent to the free carboxyl group is not essential for lymphopeptidase action. This result is consonant with the fact that lymphopeptidase hydrolyzes tripeptides only at the peptide bond next to the free amino group.

TABLE VI

Specificity of Lymphopeptidase

The rates of hydrolysis are given in terms of proteolytic coefficients, since the more resistant substrates required larger enzyme concentrations for accurate determination of the extent of hydrolysis.

Substrate	pH	C^0*
Diglycylglycine	7.9	41
Glycylglycine	7.9	0
Glycylglycinamide	8.0	0.2
Diglycylglycinamide	7.6	0.3
Triglycylglycine	7.7	0.7
Glycylglycylsarcosine	7.9	27
Glycylglycyl-L-proline	7.9	29
Carbobenzoxydiglycylglycine	7.6	0
L-Alanylglycylglycine	7.8	43
D-Alanylglycylglycine	7.6	0.1
L-Alanylsarcosylglycine	8.0	0
L-Leucylglycylglycine	7.9	46
D-Leucylglycylglycine	7.7	0
L-Leucylglycine	8.0	0.1
Carbobenzoxy-L-leucylglycylglycine	7.8	0
Glycyl-L-leucylglycine	7.9	40
Glycyl-D-leucylglycine	7.9	0.1
Glycyl-L-leucine	7.9	0.1
Glycylglycyl-L-leucine	7.7	16.5
Glycylglycyl-D-leucine	7.6	3.8
Glycylglycyl-L-leucylglycine	7.6	0.8
Glycylglycyl-L-glutamylglycine	7.8	0.2
Glutathione	8.0	0.1

* C^0 = the average K^0 per mg. of protein nitrogen.

The data in Table V show that D-LGG and D-AGG are resistant to enzyme action, thus giving evidence of stereochemical specificity with respect to the amino acid residue bearing the free amino group. Furthermore, glycyl-D-leucylglycine is not split appreciably by lymphopeptidase, in contrast to the behavior of the corresponding L peptide. In addition, glycylglycyl-D-leucine is hydrolyzed much more slowly than its L isomer, thus showing that the configuration of the amino acid bearing the free carboxyl group is also decisive for lymphopeptidase action. This stereochemical specificity with respect to the terminal amino acid at the carboxyl end of the tripeptide is in accord with the view that, in the hydrolysis of the sensitive peptide bond, the free carboxyl group is essential for the proper mutual alignment of the substrate molecule and the "active center" of the enzyme (8).

TABLE VII

Effect of Added Substances on Lymphopeptidase Activity

Enzyme concentration, 0.01 mg. of protein nitrogen per cc. of test solution; pH 7.8 to 7.9.

Added substance	K^0	
	L-Leucylglycylglycine	Diglycylglycine
None	0.44	0.39
0.01 M cysteine	0.42	0.38
0.005 M cyanide	0.43	0.38
0.005 " sulfide	0.42	0.39
0.001 " iodoacetate	0.45	0.40
0.001 " $MnSO_4$	0.43	0.37
0.001 " $ZnSO_4$		0.37
0.001 " $MgSO_4$		0.40
0.001 " $CoCl_2$		0.40
0.001 " $NiSO_4 \cdot (NH_4)_2SO_4$		0.38
0.001 " $Al_2SO_4 \cdot K_2SO_4$		0.38
0.001 " $CdSO_4$		0.16

The data presented above indicate that an exopeptidase may require, in its substrates, not only a terminal α-amino or α-carboxyl group in adjacence to the sensitive peptide bond but also additional polar groups at suitable positions in its substrate. Although lymphopeptidase appears to be the first instance of such an enzyme, the possibility exists that other cases of this kind may be found in the course of further investigation.

Other Properties of Lymphopeptidase—The action of lymphopeptidase is not inhibited appreciably by the presence of 0.01 M cysteine, 0.005 M cyanide, 0.0005 M sulfide, or 0.001 M iodoacetate (*cf.* Table VII). As noted in Table II, the addition of manganese ions does not influence markedly the enzymatic action of purified lymphopeptidase on GGG, although, in crude thymus extracts, the presence of $MnSO_4$ causes a slight inhibition of the hydrolysis of this substrate. A number of metal ions other than manganese were also tested for their effect on the rate of hydrolysis of GGG by lymphopeptidase (*cf.* Table VI). Of these, only the presence of cadmium ions resulted in a change in the rate, under the conditions of these experiments. The evaluation of this finding must await the results of further studies.

DISCUSSION

Attention has been drawn (9) to the similarity in the properties of the "aminopeptidase" of intestinal mucosa, which has been extensively purified by Ågren (10), and the tripeptide-splitting enzymes found in extracts of skin, lung, and muscle and in serum and lymph. The available data on the specificity, pH stability, and activation behavior of the comparable enzymes from these various sources are analogous to those described above for lymphopeptidase. It would appear to be a justifiable working hypothesis, therefore, to consider all these enzymatic actions to be due to an enzyme derived from lymphoid cells, although further comparison of highly purified preparations of the tripeptidases from various tissues is necessary before this view can be asserted with confidence. The results obtained to date, however, give added emphasis to the concept developed at the turn of the century by Metchnikoff (11) and others which assigns to lymphoid cells a significant rôle in the proteolytic mechanisms of body tissues and fluids. The manner in which the tripeptide-splitting lymphopeptidase may participate in protein metabolism is a problem for future study. It has already been suggested (12) that the intestinal peptidase which hydrolyzes L-AGG is identical with the "intrinsic factor" of Castle.

Examination of the available data (1, 4) also shows that crude extracts of all the various tissues mentioned above contain, in addition to tripeptidase activity, leucine aminopeptidase, and prolidase. The parallel occurrence of these three enzymes in a variety of tissues may be taken to suggest their common origin in lymphoid cells.

EXPERIMENTAL

Partial Purification of Lymphopeptidase—600 gm. of calf thymus were minced with 1.2 liters of 2 per cent sodium chloride solution in a Waring blendor; 50 cc. of toluene were added, and the mixture was stirred mechanically for 2 hours at room temperature. The suspension was strained through fine cheese-cloth to give the crude extract used for the experiments reported in Table I.

The crude extract (1460 cc.) was diluted with 4 volumes of water and heated to 50° for 1 hour with vigorous mechanical stirring. The suspension was filtered by gravity in the cold room through fluted filters. To the clear filtrate (4.6 liters) there were added, with mechanical stirring, 1115 gm. of ammonium sulfate. The precipitate which separated was removed by suction with the aid of Hyflo Super-Cel. To the filtrate (4.94 liters), 1015 gm. of ammonium sulfate were added with stirring. The precipitate was collected by filtration through hardened filter paper. The moist filter cake weighed 6.5 gm.

To prepare a solution of lymphopeptidase, 1 gm. of the filter cake was dissolved in 50 cc. of water and dialyzed in a rocking dialyzer against distilled water for 72 hours at 4°. The precipitate which separated during dialysis was removed by filtration through fluted filter paper. A few drops of toluene were added as a preservative. If kept in the refrigerator when not in use, the enzyme solution retains its lymphopeptidase activity for 2 to 3 weeks.

Measurement of Enzyme Activity—In all cases, the concentration of the synthetic substrates was 0.05 mM per cc. of test solution. The pH was maintained by means of 0.02 M veronal buffer at pH 6.8 to 8.7 and with 0.02 M citrate buffer at pH 5.0 to 5.4. The flasks containing substrate, buffer, and enzyme were kept in a water thermostat at 39°. The rate of hydrolysis was followed by measurement of the liberation of carboxyl groups by the method of Grassmann and Heyde (13). The data in Tables I to VI are given in terms of percentage of the carboxyl groups expected from the complete hydrolysis of one peptide linkage.

Glycylglycylsarcosine

Carbobenzoxyglycylglycylsarcosine Benzyl Ester—To an ethereal solution of sarcosine benzyl ester (prepared in the usual manner from 2 gm. of the hydrochloride), there was added a solution of 2 gm. of carbobenzoxyglycylglycinazide in ethyl acetate. On standing overnight at room temperature, 1.4 gm. of the coupling product separated; m.p., 110–111°. The filtrate was washed with dilute hydrochloric acid, dilute bicarbonate solution, and water, dried over Na_2SO_4, and concentrated to a small volume under reduced pressure. A second crop (0.2 gm.) of the product was obtained by the addition of petroleum ether.

$C_{22}H_{25}O_6N_3$ (427.4). Calculated, N 9.8; found, N 9.6

Glycylglycylsarcosine—1 gm. of the carbobenzoxytripeptide benzyl ester was hydrogenated in methanol in the presence of palladium black. The peptide separated during the hydrogenation and was dissolved by the addition of water. The filtrate from the catalyst was evaporated, and on addition of alcohol, the peptide crystallized. Yield, 0.35 gm. The substance was dried at 100° *in vacuo* over phosphorus pentoxide.

$C_7H_{13}O_4N_3$. Calculated. C 41.4, H 6.4, N 20.7
203.2 Found. " 41.5, " 6.3, " 20.5

Glycylglycyl-L-leucine

Carbobenzoxyglycylglycyl-L-leucine Methyl Ester—To a solution of L-leucine methyl ester (prepared from 2.5 gm. of the hydrochloride) in ethyl acetate, there were added 2 gm. of carbobenzoxyglycylglycinazide. The reaction mixture was left at room temperature overnight and then washed with dilute hydrochloric acid, dilute bicarbonate solution, and water. The ethyl acetate solution was dried over Na_2SO_4 and concentrated to a small volume under reduced pressure. The addition of ether and petroleum ether gave a crystalline precipitate. After recrystallization from ethyl acetate-petroleum ether, the substance melted at 93–94°. Yield, 1.4 gm.

$C_{19}H_{27}O_6N_3$ (393.4). Calculated, N 10.7; found, N 10.4

Glycylglycyl-L-leucine—1 gm. of the carbobenzoxytripeptide ester was dissolved in 25 cc. of methanol, and 3.8 cc. of N NaOH were added. After 20 minutes, the solution was acidified and concentrated under reduced pressure. The carbobenzoxytripeptide which crystallized was collected, washed with water, and dried over phosphorus pentoxide. It was then hydrogenated in the usual manner to yield 0.5 gm. of the desired tripeptide. For analysis, the product was recrystallized from ethanol-water and dried *in vacuo* at 100° over phosphorus pentoxide.

$C_{10}H_{19}O_4N_3$. Calculated. C 49.0, H 7.7, N 17.1
245.3 Found. " 49.2, " 7.5, " 16.9
$[\alpha]_D^{26} = -28.0°$ (2% in water)

Glycylglycyl-D-leucine

Carbobenzoxyglycylglycyl-D-leucine Methyl Ester—This compound was prepared in the same manner as was the L form; m.p., 95–96°.

$C_{19}H_{27}O_6N_3$ (393.4). Calculated, N 10.7; found, N 10.5

Glycylglycyl-D-leucine—This compound was prepared from the above carbobenzoxytripeptide ester in the same manner as was the L form.

$C_{10}H_{19}O_4N_3$. Calculated. C 49.0, H 7.7, N 17.1
245.3 Found. " 49.1, " 7.7, " 17.0
$[\alpha]_D^{26} = +27.5°$ (2% in water)

Glycyl-D-leucylglycine—This compound was prepared in the same manner as was the L form (14).

$C_{10}H_{19}O_4N_3$. Calculated. C 49.0, H 7.7, N 17.1
245.3 Found. " 49.2, " 7.5, " 16.9
$[\alpha]_D^{26} = +42.6°$ (2% in water)

Diglycylglycinamide Acetate

Carbobenzoxydiglycylglycine Ethyl Ester—This compound was prepared by the coupling of carbobenzoxyglycylglycinazide (1.5 gm.) with glycine ethyl ester (prepared from 2.5 gm. of the hydrochloride) by the procedure described above for carbobenzoxyglycylglycyl-L-leucine methyl ester. Yield, 1.4 gm.; m.p., 165°.

$C_{16}H_{21}O_6N_3$ (351.3). Calculated, N 12.0; found, N 11.9

Carbobenzoxydiglycylglycinamide—1 gm. of the above ester was dissolved in 25 cc. of methanol previously saturated with dry ammonia at 0°, and the solution was allowed to stand at room temperature for 2 days. During this period, the amide separated from the solution. Yield, 0.9 gm. The substance was recrystallized from hot water; m.p., 220°.

$C_{14}H_{18}O_5N_4$ (322.3). Calculated, N 17.4; found, N 17.1

Diglycylglycinamide Acetate—0.5 gm. of the carbobenzoxytripeptide amide was hydrogenated in methanol containing 1.0 cc. of glacial acetic acid. On concentration of the filtrate, the product crystallized. It was recrystallized from absolute alcohol. Yield, 0.3 gm.

$C_6H_{12}O_3N_4 \cdot C_2H_4O_2$.	Calculated.	C 38.7, H 6.5, N 22.6
248.2	Found.	" 39.0, " 6.4, " 22.3

SUMMARY

Calf thymus extracts contain a peptidase which hydrolyzes tripeptides at the peptide linkage adjacent to the free amino group of the substrate. This enzyme, named lymphopeptidase, has been purified appreciably by fractional precipitation with ammonium sulfate and by dialysis against water. The specificity of the purified lymphopeptidase has been studied by means of a series of peptides and peptide derivatives closely related to the substrates for this enzyme, and the results indicate that it should be classified as an "aminoexotripeptidase." The fact that the sera as well as the extracts of various tissues from several animals exhibit peptidase activity closely similar to that of lymphopeptidase suggests that this enzyme is largely responsible for the tripeptide-splitting activity of these sera and tissue extracts.

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PREPARATION OF D- AND L-METHIONINE FROM DL-METHIONINE BY ENZYMATIC RESOLUTION*

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It has been shown previously (1, 2) that the synthesis of CO–NH linkages by papain as well as by other proteinases is characterized by extreme stereochemical specificity. Thus, upon the addition of cysteine-activated papain to a mixture of carbobenzoxy-DL-glutamic acid and aniline, there is formed carbobenzoxy-L-glutamic acid anilide, which separates in crystalline form. The carbobenzoxy-D-glutamic acid which remains in solution may then be hydrogenated to yield D-glutamic acid (2).

As noted elsewhere (3), it appears desirable to examine the feasibility of this enzymatic resolution method for the preparation of the optically active isomers of several amino acids (methionine, threonine, valine, and isoleucine) whose DL forms are now readily accessible by synthesis. In the present communication there is described the preparation, by the enzymatic resolution of DL-methionine, of D- and of L-methionine. A method for the resolution of DL-methionine has been described previously by Windus and Marvel (4), who used the classical procedure of Emil Fischer. Duschinsky and Jeannerat (5) prepared L-methionine from the racemate by the selective oxidation of the D isomer with D-amino acid oxidase.

When carbobenzoxy-DL-methionine is incubated with aniline in the pres ence of cysteine-activated papain, carbobenzoxy-L-methionine anilide crystallizes with a yield of 95 per cent. From the filtrate, there may be isolated carbobenzoxy-D-methionine which, on catalytic hydrogenation, is converted to D-methionine of satisfactory purity.

It was intended to obtain L-methionine by the hydrolysis of carbobenzoxy-L-methionine anilide with hydrochloric acid. The main product of the cleavage was found to be not L-methionine, however, but S-benzyl-L-homocysteine. The formation of this compound from carbobenzoxy-L-methionine anilide may be explained by assuming the formation of benzyl chloride in the course of the acid hydrolysis of the carbobenzoxy group, followed by the conversion of the thioether to a sulfonium ion. The preferential elimination of the methyl group during the decomposition of the

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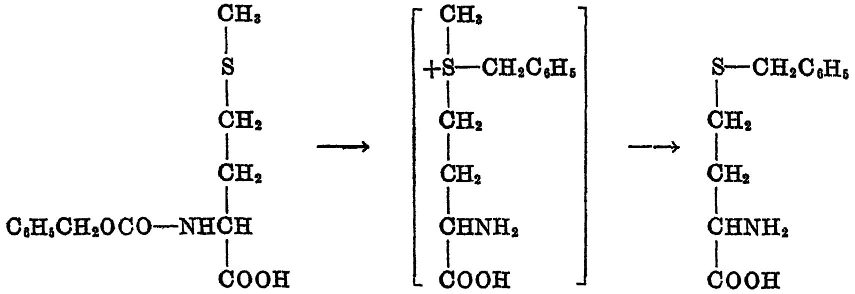
sulfonium ion would then lead to the formation of S-benzyl-L-homocysteine.



Support for this interpretation comes from the observation that, if DL-methionine is refluxed with benzyl chloride and hydrochloric acid, S-benzyl-DL-homocysteine is formed.

The application of the enzymatic resolution method thus offers an additional method for the preparation of S-benzyl-L-homocysteine. This compound was first obtained by du Vigneaud and Patterson (6) from DL-methionine by the conversion of the latter substance to DL-homocystine, which was then reduced with sodium in liquid ammonia. Treatment with benzyl chloride gave S-benzyl-DL-homocysteine which was resolved by fractional crystallization of the brucine salts of the N-formyl derivatives. Du Vigneaud and Patterson prepared D- and L-homocysteine from the optically active S-benzyl derivatives by treatment with sodium in liquid ammonia.

The difficulty encountered in the attempted preparation of L-methionine from carbobenzoxy-DL-methionine may be avoided if, in place of the carbobenzoxy derivative, the benzoyl compound is employed in the enzymatic resolution. In the presence of papain, benzoyl-L-methionine anilide is formed in 97 per cent yield and pure L-methionine may be prepared by acid hydrolysis of this derivative. The isolation of pure benzoyl-D-methionine from the filtrate of the anilide proved to be difficult, in contrast to the situation found in the case of the corresponding carbobenzoxy compound. Hydrolysis of the impure benzoyl-D-methionine gave partially racemic preparations of the amino acid.

The ready availability of the optically active isomers of methionine makes possible their use in the synthesis of peptides of L- and of D-methionine. The preparation of such peptides will be the subject of a subsequent communication.

EXPERIMENTAL

Carbobenzoxy-DL-methionine—To a solution of 30 gm. (0.2 mole) of DL-methionine in 100 cc. of 2 N NaOH, there were added 37.5 gm. (0.22 mole) of carbobenzoxy chloride and 120 cc. of 2 N NaOH in five portions over a period of 30 minutes. The reaction mixture was kept at 0° and stirred continuously during this time. After further stirring for 1 hour at room temperature, the solution was acidified to Congo red with concentrated hydrochloric acid, causing the precipitation of crystalline carbobenzoxy-DL-methionine. The crystals were collected and dried over phosphorus pentoxide *in vacuo*. Yield, 50 gm. (88 per cent); m.p., 110–112°. After recrystallization from 40 per cent ethanol, the substance melted at 112°.

$C_{13}H_{17}O_4NS$.	Calculated.	C 55.1, H 6.1, N 4.9
283.2	Found.	" 55.2, " 6.0, " 4.9

Enzymatic Synthesis of Carbobenzoxy-L-methionine Anilide—14.5 gm. (0.05 mole) of carbobenzoxy-DL-methionine were dissolved in 50 cc. of N NaOH and added to 9.5 cc. (0.11 mole) of aniline. A solution of 0.6 gm. of cysteine hydrochloride in 20 cc. of water was added, followed by 40 cc. of 0.2 M citrate buffer (pH 5.0). A solution obtained by the extraction of 3 gm. of crude papain (dried papaya latex) with 40 cc. of water was then introduced and the reaction mixture was diluted to 250 cc. with water. Separation of the anilide began immediately, crystallization being induced by vigorous shaking of the flask. The reaction mixture was placed in a constant temperature bath at 38° for 4 days. The anilide which separated was removed daily and the filtrate was replaced in the bath after adjusting the pH to 5 with a few drops of concentrated hydrochloric acid. The combined precipitates weighed 8.7 gm. (95 per cent). The compound was recrystallized from 40 per cent ethanol; m.p., 162.5°.

$C_{19}H_{22}O_3N_2S$.	Calculated.	C 63.6, H 6.2, N 7.8
358.3	Found.	" 63.5, " 6.2, " 8.1

$[\alpha]_D^{29} = -14.8°$ (1.45% in glacial acetic acid)

Carbobenzoxy-D-methionine—The filtrate remaining from the enzymatic synthesis of carbobenzoxy-L-methionine anilide was heated to boiling to coagulate the proteins and was decolorized with a few gm. of Darco. The clear filtrate was acidified to Congo red with concentrated hydrochloric acid. The syrup which separated crystallized after being kept in the icebox for 24 hours. The crystals were dissolved in ether and the ethereal solution was extracted with an aqueous solution of potassium bicarbonate. Upon acidification of the aqueous layer, there separated crystalline carbobenzoxy-D-methionine which was collected and washed with cold water. Yield, 7 gm. (85 per cent). After recrystallization from 50 per cent ethanol, the substance melted at 69–70°.

$C_{13}H_{17}O_4NS$.	Calculated.	C 55.1, H 6.1, N 4.9
283.2	Found.	" 54.9, " 6.1, " 4.8

D-*Methionine*—2.85 gm. (0.01 mole) of carbobenzoxy-D-methionine were dissolved in 20 cc. of methanol containing a few drops of glacial acetic acid and hydrogenated with palladium black as the catalyst. The hydrogenation required 11 hours and fresh catalyst was added at the end of 5 hours. 50 cc. of hot water were added to dissolve the free amino acid, and the catalyst was removed by filtration and washed on the filter with more hot water. The filtrate and washings were concentrated to dryness *in vacuo* and the residue was extracted with ether to remove any unchanged carbobenzoxy-D-methionine. The ether-insoluble material was recrystallized from 75 per cent ethanol. The crystalline D-methionine was collected, washed with small amounts of absolute alcohol and ether, and dried over phosphorus pentoxide. Yield, 1.0 gm. (67 per cent).

$C_5H_{11}O_2NS$. Calculated. C 40.2, H 7.5, N 9.4
149.2 Found. " 40.1, " 7.4, " 9.1
$[\alpha]_D^{27} = -21.5°$ (1.2% in 0.2 N hydrochloric acid)

Windus and Marvel (4) report $[\alpha]_D^{25} = -21.18°$ (0.8 per cent in 0.2 N hydro chloric acid).

*S-Benzyl-*L*-homocysteine*—2.5 gm. (0.0067 mole) of carbobenzoxy-L-methionine anilide were refluxed with 20 cc. of concentrated hydrochloric acid for 10 hours. The hydrolysate was extracted with ether and the aqueous layer was concentrated under reduced pressure. The concentrate was neutralized with saturated lithium hydroxide solution, yielding a heavy white precipitate which was collected and washed with small amounts of water, alcohol, and ether. The product was recrystallized from boiling water. Yield, 0.5 gm. (30 per cent); m.p., 243–244° (with decomposition). Du Vigneaud and Patterson (6) report 247–252° for S-benzyl-D-homocysteine.

$C_{11}H_{15}O_2NS$. Calculated. C 58.6, H 6.7, N 6.2
225.2 Found. " 58.6, " 6.7, " 6.1
$[\alpha]_D^{26} = +27.2°$ (1% in N hydrochloric acid)

Du Vigneaud and Patterson (6) found for S-benzyl-D-homocysteine $[\alpha]_D^{26} = -25°$ (1 per cent in N hydrochloric acid).

*S-Benzyl-*DL*-homocysteine*—1.5 gm. (0.01 mole of DL-methionine) were dissolved in 20 cc. of concentrated hydrochloric acid, 1.4 cc. (0.012 mole) of benzyl chloride were added, and the mixture was refluxed for 11 hours. The reaction mixture, after being cooled, was extracted with ether, and the aqueous layer was concentrated to a syrup under reduced pressure. The residue was taken up in 30 cc. of water and neutralized with saturated lithium hydroxide solution. The resulting precipitate was collected and washed successively with cold water, ethanol, and ether. Yield, 1.0 gm. (45 per cent). After recrystallization from boiling water, the substance melted with decomposition at 240–243°. Du Vigneaud and Patterson (6) report 240–250°.

$C_{11}H_{15}O_2NS$ (225.2). Calculated, N 6.2; found, N 5.9

Benzoyl-DL-*methionine*—This compound was prepared according to the directions of Steiger (7) for the benzoylation of amino acids. 32 gm. of the benzoyl derivative were obtained from 20 gm. of DL-methionine, representing a yield of 95 per cent. The crude material melted at 145–150°. After recrystallization from 33 per cent ethanol, the compound melted sharply at 151°. This value agrees with that reported by Hill and Robson (8).

Enzymatic Synthesis of Benzoyl-L-*methionine Anilide*—19.45 gm. (0.077 mole) of benzoyl-DL-methionine were dissolved in 77 cc. of N NaOH and 13.9 gm. (0.154 mole) of aniline were added. A solution of 0.93 gm. of cysteine hydrochloride in 25 cc. of water was then added, followed by 40 cc. of 0.2 M citrate buffer (pH 5.0). The enzyme solution, prepared by extracting 4.6 gm. of papain with 62 cc. of water, was introduced, and the mixture was agitated and placed in a constant temperature bath at 38°. Another 190 cc. of buffer and more water were added gradually over a period of 2 hours, making the total volume 750 cc. After 18 hours, the benzoyl-L-methionine anilide which had separated was collected and the filtrate was replaced in the bath. The first crop weighed 11.3 gm. (90 per cent). Another 0.9 gm. was collected after the next 24 hours, giving a total yield of 12.2 gm. (97 per cent). The compound was purified by recrystallization first from ethyl acetate and then from ethanol-water; m.p., 159°.

$C_{18}H_{20}O_2N_2S$.	Calculated.	C 65.8, H 6.2, N 8.5
328.3	Found.	" 65.8, " 6.2, " 8.2

Acid Hydrolysis of Benzoyl-L-*methionine Anilide*—2.2 gm. (0.0067 mole) of benzoyl-L-methionine anilide were suspended in 150 cc. of 6 N hydrochloric acid and refluxed on a sand-bath for 12 hours. After cooling the mixture, the benzoic acid which had crystallized was removed by filtration. The filtrate was concentrated under reduced pressure to 50 cc. and then was extracted with two 35 cc. portions of ether. The aqueous layer was concentrated and the residue was taken up in 15 cc. of absolute alcohol. The addition of 5 cc. of pyridine caused the crystallization of L-methionine. The crystals were collected and washed successively with absolute alcohol and ether. Yield, 0.5 gm. (50 per cent). The rotation of the unrecrystallized material was $[\alpha]_D^{24} = +20.7°$ (1 per cent in 0.2 N hydrochloric acid). After recrystallization from 75 per cent ethanol, the optical activity was unchanged. The substance melted at 281° (corrected) with previous darkening and shrinkage. Mueller (9) reports a melting point of 283° for L-methionine.

$C_5H_{11}O_2NS$.	Calculated.	C 40.2, H 7.5, N 9.4
149.2	Found.	" 40.0, " 7.3, " 9.1

Acid Hydrolysis of Benzoyl-D-methionine—The filtrate from the enzymatic synthesis of benzoyl-L-methionine anilide was heated to boiling to coagulate the proteins, was decolorized with Darco and filtered, and the filtrate was concentrated *in vacuo* to 185 cc. After acidification to Congo red, the benzoyl-D-methionine separated as an oil which crystallized readily upon stirring and scratching. The crude crystals were collected, washed with a small amount of cold water, and dried over phosphorus pentoxide and sodium hydroxide. Yield, 9.4 gm. (96 per cent). Partial purification was effected by the extraction of an ethyl acetate solution of this material with aqueous potassium bicarbonate, and acidification of the bicarbonate solution. The crystalline material which resulted was washed and dried as before. Further purification by recrystallization was unsuccessful because the solubility of benzoyl-D-methionine proved to be greater than that of benzoyl-DL-methionine. Therefore, 2.55 gm. (0.01 mole) of the partially purified benzoyl-D-methionine were suspended in 250 cc. of 10 per cent hydrochloric acid and refluxed on a sand-bath for 12 hours. The benzoic acid which had separated was removed by filtration, and the filtrate was concentrated under reduced pressure to a volume of 125 cc. After extraction with two 60 cc. portions of ether, the solution was further concentrated *in vacuo* and the residue was taken up in 18 cc. of absolute alcohol. The alcoholic solution was clarified by filtration and the methionine was precipitated by the addition of 4 cc. of pyridine. Yield, 1.0 gm. (67 per cent). The optical rotation of the product was $[\alpha]_D^{26} = -16.7°$ (1.7 per cent in 0.2 N hydrochloric acid). Recrystallization of 0.9 gm. of the above material from 100 cc. of 75 per cent ethanol yielded 0.6 gm. of methionine with a rotation of $[\alpha]_D^{26} = -15.7°$ (0.7 per cent in 0.2 N hydrochloric acid), thus indicating that the material was being enriched with respect to DL-methionine rather than D-methionine.

An additional fraction (0.16 gm.) was recovered from the mother liquor, and this had a rotation of $[\alpha]_D^{25} = -20.7°$ (0.8 per cent in 0.2 N hydrochloric acid).

$C_5H_{11}O_2NS$.	Calculated.	C 40.2, H 7.5, N 9.4
149.2	Found.	" 40.4, " 7.5, " 9.2

Although reasonably pure D-methionine was obtained, the procedure was less satisfactory than that involving the hydrogenation of carbobenzoxy-D-methionine.

SUMMARY

DL-Methionine has been resolved by the action of papain on a mixture of carbobenzoxy-DL-methionine and aniline. The anilide of the L isomer separates in crystalline form, and from the filtrate there may be isolated carbo-

benzoxy-D-methionine which upon catalytic hydrogenation is converted to D-methionine. Acid hydrolysis of carbobenzoxy-L-methionine anilide yields S-benzyl-L-homocysteine. L-Methionine may be prepared by the enzymatic synthesis of benzoyl-L-methionine anilide from benzoyl-DL-methionine and aniline, followed by acid hydrolysis of the anilide.

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THE UTILIZATION OF PEPTIDES BY LACTIC ACID BACTERIA*

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The extensive use of the lactic acid bacteria for the microbiological assay of amino acids has provided valuable data on the amino acid composition of proteins (1). In some cases, attempts have been made to determine the amino acid content of materials such as plasma (2), urine (3), and partial hydrolysates of tissue proteins (4) without reference to the question as to whether these materials contained peptides of the amino acid under test and whether these peptides were utilized by the test organism as effectively as the unsubstituted amino acid. Subsequent work (5) showed, indeed, that acid hydrolysis of such complex materials markedly raised their apparent amino acid content. The experiments described in the present communication indicate that there exists considerable variation in the utilization of leucine peptides by *Lactobacillus arabinosus* and by *Streptococcus faecalis*. Similar experiments have been reported recently by Ågren (6), who found differences in the utilization of various leucine and valine peptides (of unstated configuration) by several lactic acid bacteria. In addition, it has been shown previously (7) that the *leucineless* mutant of *Escherichia coli* exhibits considerable selectivity in the utilization of leucine peptides for growth. The suggestion has been made (7, 8) that, in the case of many peptides, utilization for growth is preceded by enzymatic hydrolysis to yield the essential amino acid. Differences in the effectiveness of such peptides in replacing an essential amino acid may be taken, therefore, to reflect differences in the rate of cleavage of the peptides by the bacterial peptidases.

Recent work has shown, however, that some peptides may be more effective in promoting bacterial growth than are the component amino acids. This has been brought out strikingly by the work of Woolley (9) on "strepogenin," and Womack and Rose (10) have raised the question as to the possible identity of this material with a peptide-like factor, which they have found to promote the growth of the rat. Moreover, data have been obtained[1] to show that, in the case of a *prolineless* mutant of *Escherichia coli*, certain proline peptides are more effective growth factors than is proline itself. It is clear, therefore, that there exist particular combinations of amino acids bound in peptide linkage which may be utilized in metabolism without prior enzymatic hydrolysis.

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[1] Simmonds, S., and Fruton, J. S., unpublished experiments.

Testing Methods—The microorganisms used were *Lactobacillus arabinosus* 8014,[2] *Lactobacillus casei* 7469,[2] *Streptococcus faecalis* 9790,[2] and *Leuconostoc mesenteroides* P-60 8042.[2] Stock cultures of the organisms were carried in a medium containing 2 per cent Difco yeast extract, 0.5 per cent glucose, 2 per cent sodium acetate, and 1.5 per cent agar. The components of the basal medium differ for the most part only quantitatively from those usually employed, but because uniform success has been attained with this medium for all of the lactic acid bacteria listed above, its composition is presented in Table I.

TABLE I

Composition of Complete Basal Medium

Component	Amount per 10 cc. final medium	Component	Amount per 10 cc. final medium
	mg.		*mg.*
Amino acid mixture		Salts C* (0.2 cc. per 10 cc. of final medium)	
L-Asparagine	4		
L-Glutamic acid	5	$MgSO_4 \cdot 7H_2O$	8.0
L-Lysine·HCl	2	NaCl	0.4
L-Cystine	1	$FeSO_4 \cdot 7H_2O$	0.4
L-Arginine·HCl	1	$MnSO_4 \cdot 4H_2O$	1.6
L-Histidine·HCl·H_2O	1	Glucose	200
L-Proline	1	Purines and pyrimidines	γ
L-Tyrosine	1	Adenine sulfate·$2H_2O$	100
L-Serine	1	Guanine·HCl·$2H_2O$	100
L-Leucine†	1	Uracil	100
DL-Tryptophan	1	Xanthine	100
DL-Alanine	2	Vitamins	
DL-Threonine	2	Biotin	0.05
DL-Isoleucine	2	Folic acid	0.05
DL-Valine	2	*p*-Aminobenzoic acid	0.5
DL-Methionine	2	Thiamine·HCl	10.0
DL-Phenylalanine	2	Riboflavin	10.0
Glycine	1	Pyridoxine·HCl	10.0
$(NH_4)_2SO_4$	30	Nicotinic acid	10.0
Sodium acetate	200	Calcium pantothenate	10.0
KH_2PO_4	25		
K_2HPO_4	25		

* Roberts and Snell (11).

† Omitted in the assay for this amino acid and for leucine derivatives.

The procedure in conducting the microbiological assay is similar to that generally employed (12). Prior to use, the inocula were diluted 1:30 or 1:60, as indicated, and stock cultures were transferred to fresh agar each week. All assay tubes were incubated at 37° and removed at suitable intervals for turbidimetric measurement with the Evelyn photoelectric colorimeter (filter No. 620) or for the estimation of the acid production by titration with 0.1 N NaOH.

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[2] American Type Culture Collection, Georgetown University, Washington, D. C.

Utilization of Leucine Peptides by Lactic Acid Bacteria—It has been reported previously (13) that D-leucine is not used by *Lactobacillus arabinosus* for growth (as measured by acid production after an incubation period of 72 hours) and that this amino acid does not inhibit the activity of L-leucine. This result has been confirmed in the present studies and it has also been found that the same is true for *Streptococcus faecalis* (*cf.* Table II). It was noted, however, that of two commercial preparations of DL-leucine only one gave growth responses which were to be expected on the basis of its L-leucine content. The other preparation of DL-leucine consistently gave lower values and it may be concluded, therefore, that this material was impure.[3] This finding further emphasizes the need for careful checking of the purity of the standards used for the microbiological assay of amino acids.

In evaluating the utilization of the leucine peptides, it was desirable to estimate the extent of bacterial growth at different time intervals, in addition to the customary determination of the acid production after 72 hours. This was done by measurement of turbidity after 16 and 40 hours, and the data in Table III represent the growth response to the addition of L-leucine. In order to have a common basis for the comparison of the utilization of the leucine peptides, the concentration levels at which they were tested were such that the amount of leucine they could yield on complete hydrolysis fell within the range of the leucine concentrations given in Tables II and III. The relative growth-promoting activity of each leucine derivative could then be expressed as per cent of that to be expected if all the leucine in the compound were available for growth. In Table IV, the values for the "per cent leucine activity" are given as averages of duplicate determinations when these agreed within reasonable limits. It will be noted that, in two cases, such agreement was not obtained.

The data presented in Table IV show that all of the peptides of L-leucine which were tested served as growth factors for the two organisms employed in these studies. It will be noted, however, that even at 72 hours utilization in the case of most of the peptides was less than that expected if all of the peptide-bound leucine were available for growth. Moreover, there was found appreciable variation in the response of the two organisms to individual peptides such as glycyl-L-leucine. Although certain of the peptides, *e.g.*, L-leucylglycylglycine, produced a similar growth response relative to

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[3] A similar result was obtained with the *leucineless* mutant of *Escherichia coli* (Simmonds, S., and Fruton, J. S., unpublished experiments).

TABLE II

Acid Production by Lactic Acid Bacteria in Presence of Leucine

Leucine preparation added to basal medium	Amount per tube*	0.1 N acid formed per tube after 72 hrs.	
		Lactobacillus arabinosus	*Streptococcus faecalis*
	γ	*cc.*	*cc.*
L-Leucine†	0	0.2	0.3
	20	3.4	2.4
	40	6.2	4.5
	60	8.4	6.4
	80	10.4	7.4
	100	12.6	8.0
" + D-leucine†	0	0.2	0.2
	20	3.3	2.3
	40	6.0	4.4
	60	8.3	6.2
	80	10.1	7.2
	100	12.2	8.1
DL-Leucine‡	0	0.2	0.3
	20	3.6	2.1
	40	6.2	4.3
	60	8.4	6.1
	80	10.6	7.1
	100	12.6	7.9
" §	0	0.1	0.2
	20	0.8	0.8
	40	1.8	1.7
	60	3.2	2.6
	80	4.1	3.4
	100	4.9	4.4

* Refers to amount of L-leucine added.

† Obtained through the courtesy of Dr. William H. Stein.

‡ Merck preparation.

§ Eastman preparation.

TABLE III

Growth of Lactic Acid Bacteria in Presence of Leucine

Amount of L-leucine per tube	Galvanometer reading.			
	Lactobacillus arabinosus		*Streptococcus faecalis*	
	16 hrs.	40 hrs.	16 hrs.	40 hrs.
γ				
0	94	93	98	94
20	83	73	88	83
40	74	56	80	74
60	64	42	72	66
80	58	36	65	58
100	51	32	61	51

the leucine standard at 16, 40, and 72 hours, in the case of other peptides, notably L-leucyl-L-tyrosine and diglycyl-L-leucylglycine, the time of incubation was an important factor in determining the extent of bacterial growth. These findings indicate that the growth-promoting activity of L-leucine peptides depends upon the position of the leucine residue with respect to the other amino acid residues, as well as upon the nature of the other amino acid residues. Furthermore, these results support the view that the rate of enzymatic hydrolysis of these peptides plays a significant rôle in their utilization by microorganisms.

TABLE IV

Effect of Leucine Derivatives on Growth of Lactic Acid Bacteria

Compound*	Growth-promoting activity†					
	Lactobacillus arabinosus			*Streptococcus faecalis*		
	16 hrs.	40 hrs.	72 hrs.	16 hrs.	40 hrs.	72 hrs.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
L-Leucylglycine	55	51	54	63	76	80
D-Leucylglycine		1.5	2		2.2	2
N-Methyl-DL-leucylglycine						
L-Leucylglycylglycine	63	66	76	58	62	57
D-Leucylglycylglycine		1.8	1.5		2	
L-Leucyl-L-tyrosine	71	86	102	92	106	93
L-Leucinamide acetate	13, 34	27	47	3.5	13	13
D-Leucinamide "						
Glycyl-L-leucine	103	99	102	63	66	72
Glycyl-D-leucine					1.7	
Diglycyl-L-leucylglycine		22	58	52	66	74
Triglycyl-L-leucylglycine‡		36	62			
Carbobenzoxyglycyl-L-leucine		18, 42	68			
Acetyl-L-leucine			6			
Acetyldehydroleucine						
Acetyldehydroleucylglycine						
Acetyldehydroleucinamide						

* The bibliographic references to the preparation of these compounds are given by Simmonds *et al.* (7).

† Expressed as per cent of that to be expected if all the leucine in the compound were available for growth. The blank indicates that no growth was noted at any concentration level tested.

‡ This substance was not tested with *Streptococcus faecalis*.

In agreement with the results obtained with the *leucineless* mutant of *Escherichia coli*, the peptides containing D-leucine were uniformly inactive, regardless of the organism used, time of incubation, or peptide concentration. It is worthy of note that the leucine derivatives L-leucinamide and carbobenzoxyglycyl-L-leucine are utilized by *Lactobacillus arabinosus*, and only the first of these substances is at all effective for *Streptococcus faecalis*. In this connection, it should be of interest to compare the amino- and carboxypeptidase activity of these two organisms. Leucine derivatives such as N-methyl-DL-leucylglycine, acetyldehydroleucine, acetyldehydroleucylglycine, and acetyldehydroleucinamide were inactive for both organisms, and only slight activity was noted for acetyl-L-leucine in the case of *Lactobacillus arabinosus*.

"Strepogenin" Activity of Peptides—Sprince and Woolley have called attention (14) to the presence, in liver extracts and in partial hydrolysates of casein, of a peptide (strepogenin) which favors the growth of *Lactobacillus casei*, and Woolley has reported further (9) that a mixture of the diastereoisomeric D- and L-serylglycyl-L-glutamic acids has strepogenin activity. In the course of the present investigation, L-serylglycyl-L-glutamic acid was synthesized by the reaction of carbobenzoxy-L-serylglycinazide with L-glutamic acid diethyl ester, followed by the saponification of the carbobenzoxytripeptide ester and the removal of the carbobenzoxy group by catalytic hydrogenation with palladium black. In addition, L-seryl-L-alanyl-L-glutamic acid was made by an analogous procedure.[4]

TABLE V

Strepogenin Activity of Various Materials

Component	Amount per 10 cc.	Galvanometer reading*			
		Lactobacillus arabinosus	*Lactobacillus casei*	*Leuconostoc mesenteroides*	*Streptococcus faecalis*
	mg.				
Basal medium		21	98	89	45
" " + liver fraction L	10	14	59	44	41
	5		75		
" " + L-serylglycyl-L-glutamic acid	8		82		
	3		90		
Basal medium + L-seryl-L-alanyl-L-glutamic acid	10		100		

* Readings made with Evelyn photoelectric colorimeter after 18 hours incubation at 37°; inoculum dilution 1:60. Uninoculated control tubes were used as blanks for setting the galvanometer at 100.

Before testing the activity of these and other peptides for strepogenin activity, it appeared desirable to determine whether only *Lactobacillus casei* required strepogenin for growth. The data in Table V show that, of the four organisms tested, only *L. casei* and *Leuconostoc mesenteroides* P-60 require a source of strepogenin, since liver fraction L[5] did not affect appreciably the growth of *L. arabinosus* or *Streptococcus faecalis*.

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[4] A detailed description of the synthesis of these tripeptides will be presented in a separate communication.

It will be noted from Table V that L-serylglycyl-L-glutamic acid exhibits a slight but unmistakable growth-promoting activity for *Lactobacillus casei*. This confirms, in a qualitative sense, the result reported by Woolley. Since 1 mg. of the tripeptide was equivalent in activity to about 0.5 mg. of the liver fraction, it is clear that the latter must contain a substance which is considerably more active than the synthetic tripeptide. It may be added that the closely related L-seryl-L-alanyl-L-glutamic acid was inactive under the conditions of our assay, as were α-L-glutamyl-L-glutamic acid, α-L-glutamylglycylglycine, diglycyl-α-L-glutamylglycine, and glycyl-α-L-glutamyl-L-tyrosine.

The authors wish to express their thanks to Miss Barbara Illingworth for valuable assistance in the course of this investigation.

SUMMARY

Experiments on the utilization of various peptides of L-leucine by *Lactobacillus arabinosus* and *Streptococcus faecalis* have shown that the growth-promoting activity of such peptides depends on the position of the leucine residue with respect to the other amino acid residues, the nature of the other amino acids, and the time of incubation.

The strepogenin activity of L-serylglycyl-L-glutamic acid for *Lactobacillus casei*, reported by Woolley, has been confirmed.

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[5] Obtained through the courtesy of Dr. David Klein, The Wilson Laboratories, Chicago, Illinois.

THE SOLUBILITY OF NITROUS OXIDE IN BLOOD AND BRAIN*

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Since the formulation of the lipide theory of anesthesia (1) there has been some interest in the solubilities of various volatile anesthetics in the brain (2–6). Unfortunately the results obtained *in vivo* have been equivocal and inexact because of the technical difficulties of obtaining and analyzing without loss samples of brain containing volatile gas. Even the studies on solubility *in vitro*, on which the theory was largely based, were made on peanut, olive, or similar oils rather than on brain lipides. Our attention was drawn to the problem of the solubility of gases in brain in the course of the development of a method for measurement of cerebral blood flow by means of the blood-brain exchange of an inert gas, nitrous oxide (7). It can be shown that cerebral blood flow may be calculated from the arterial (A) and cerebral venous (V) blood nitrous oxide concentrations over a time interval (u) measured from the onset of inhalation of a comparatively low tension of nitrous oxide. The interval u must be of sufficient length to insure practically complete equilibrium between brain and blood draining the brain with respect to mean nitrous oxide tension. If the results are to be expressed in flow per unit weight of brain rather than in terms of flow per unit of nitrous oxide capacity, a factor (S) must be introduced, representing the brain-blood partition coefficient of nitrous oxide or the ratio of nitrous oxide dissolved per gm. of brain to that dissolved per cc. of blood at a constant nitrous oxide tension and at 37°. The formula for calculation of cerebral blood flow, which is derived elsewhere may be expressed as follows:

$$\text{Cerebral blood flow} = \frac{V_u S}{\int_0^u (A - V)dt}$$

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† National Research Council Fellow in Anesthesiology.

In order to make possible the calculation of absolute values it was necessary to evaluate the partition coefficient (*S*) and the minimum time (*u*) for equilibrium between the brain and its venous blood. This was attempted by means of both *in vitro* and *in vivo* techniques.

Methods

Determination of Nitrous Oxide Solubilities in Blood and Brain in Vitro—Freshly shed, heparinized whole blood is used as such. The brain, however, is too viscid for convenient handling; a representative sample of about 10 gm. is accurately weighed and homogenized with exactly 5 cc. of distilled water in a Potter glass homogenizer (8). The homogenate is pressed through coarse gauze to remove small shreds of connective tissue and transferred to a 50 cc. glass syringe in which equilibration with nitrous oxide is to occur. All air is expelled from the syringe which is then filled with pure nitrous oxide after several flushings with the same gas. The tip of the syringe is immediately closed by means of a sealed needle hub and the syringe containing the nitrous oxide and blood or brain homogenate is rotated slowly in a water bath maintained at 37°. It is important to make sure frequently that the plunger of the syringe is free to move in the barrel to compensate for temperature-induced volume changes and that the syringe is not more than a cm. below the surface of the water of the bath. At intervals of 15 minutes the syringe is removed from the bath, the gas is expelled, and fresh nitrous oxide added for three such flushings. Equilibration is then allowed to proceed for another hour. At the end of that time, with the syringe held vertically in the bath, the gas is entirely expelled and the cap replaced. The syringe may now be removed from the bath and part of its contents analyzed for nitrous oxide as follows, after which the remainder may be reequilibrated: 5 drops of caprylic alcohol plus 3 cc. of distilled water are extracted for 3 minutes in the 50 cc. chamber of a Van Slyke-Neill manometric apparatus, then expelled as completely as possible, 1 cc. of mercury in addition being permitted to rise into the cup. The water and caprylic alcohol are removed from the surface of the mercury in the cup. A stout capillary tube of about 2 mm. bore and a cm. longer than the depth of the cup on the manometric apparatus is now securely fastened to the tip of the syringe by means of a short length of heavy plastic tubing. The free end of this capillary tube, which is somewhat tapered to resemble the tip of an Ostwald-Van Slyke pipette, is fitted with a rubber tip for sealing against the bottom of the cup. The contents of the syringe are carefully expressed to this tip and a little excess is expelled. The capillary is then held vertically, its rubber tip pressed against the bottom of the cup, and, against a slight positive pressure of mercury in the manometric apparatus, the contents of the syringe, by pressure on the plunger, are carefully forced into the chamber, stopping accurately at the 2 cc. mark. The stop-cock at the top of the chamber is now closed, the cup emptied, and the mercury in the chamber brought down to the 50 cc. mark. 6 cc. of deaerated oxygen absorber (KOH-hydrosulfite-anthraquinone reagent employed in blood oxygen analyses (9)) are now added to the cup and the lower 5 cc. admitted to the chamber in several quick additions to wash the 2 cc. volume clean. The upper cock is sealed with mercury and the contents of the chamber are extracted for about 5 minutes. The liquid is then permitted to rise smoothly to the 2 cc. mark and pressure and temperature readings are taken. Extraction at 50 cc. is repeated for 5 minute periods until the pressure reading (corrected for any temperature change) remains constant for three successive determinations. Nitrous oxide concentration is calculated as follows:

$$\text{vol. \% N}_2\text{O} = f_{\text{N}_2\text{O}}[r_a - (r_0 + C_w)]$$

where r_a = the manometric reading for the sample and r_0 = the manometric reading for the blank. This is run once for each series of analyses, and consists in deaerating 3 cc. of distilled water, expelling only 1 cc., and treating the 2 cc. left in the chamber as if it were a sample to be analyzed. C_w = the correction for change in water vapor corresponding to any temperature change between the blank and the sample for analysis. $C_w = (t_a - t_0)\ \Delta p_w$ where t_a = the temperature of the sample analysis and t_0 = the temperature of the blank analysis. Δp_w = the change in water vapor tension corresponding to 1° of temperature change in the temperature region of the analysis. $f_{\text{N}_2\text{O}}$ = the manometric factor for N_2O calculated from a factor i of 1.03 (Orcutt and Waters (10)) and values of 0.507 and 0.438 for the α' of the analysis mixture at 20° and 30° respectively.

The Bunsen solubility coefficient (α) is calculated from the N_2O concentration in volume per cent in the blood or homogenate as follows:

$$\alpha = \frac{\text{N}_2\text{O vol. \%}}{100} \times \frac{760}{B - T_w}$$

where B = the barometric pressure and T_w = water vapor tension at 37°.

In the case of the brain homogenate the α obtained above is for the brain-water mixture as a whole, but since the α for water can be determined, and the relative quantities of brain and water are known, the solubility of nitrous oxide per gm. of brain can readily be calculated.

$$\alpha_b = \frac{\alpha_h\left(\frac{W_b}{1.05} + V_w\right) - V_w\alpha_w}{W_b}$$

where α_b, α_h, α_w = the Bunsen coefficients for 1 gm. of brain, 1 cc. of homogenate, 1 cc. of distilled water respectively, all at 37°. W_b = the weight of the brain sample, V_w = the volume of water, and 1.05 = the specific gravity of brain.

Determination of Brain and Cerebral Venous Nitrous Oxide Concentrations in Vivo—These studies were performed on dogs anesthetized with sodium pentobarbital. A T-tube, attached to inspiratory and expiratory flutter valves through which gas mixtures could be administered without rebreathing, was introduced into the trachea. The skull was trephined through the occipital protuberance and a threaded brass cannula was screwed into the skull, tapping the torcular Herophili (confluence of the sinuses). A solution of heparin was used in this cannula to prevent clotting. A preliminary period of inhalation of 100 per cent oxygen for 1 hour insured practically complete denitrogenation of the brain. The animal was then permitted to breathe a mixture of 40 per cent nitrous oxide and 60 per cent oxygen. At a variable time after the beginning of nitrous oxide inhalation a sample of cerebral venous blood was collected anaerobically from the cannula in the torcular and immediately thereafter the animal was sacrificed by passage of an electric current through the thorax, a procedure which produced instantaneous ventricular fibrillation. The scalp and muscles were quickly removed from the cranium and two holes 1 inch in diameter were made by means of a trephine on opposite sides of the skull just above the zygomatic arch, with care not to cut completely through the bone. The head was then removed and immersed in a bath of water through which a fine spray of 40 per cent nitrous oxide-60 per cent oxygen had been passing for about 30 minutes. Under the surface of this bath, the trephine holes were completed and the buttons of bone removed. The dura was then cut and a sample of brain was taken anaerobically by means of the following technique. It was found possible to perform all of these operations from the sacrifice of the animal to removal of the brain sample in about 10 minutes, the brain being exposed to the water bath for only 30 seconds.

The brain sampler (Fig. 1) is simply a 10 cc. all-glass syringe in which the end of the barrel has been cut off and the rim beveled to a fair cutting edge. This end may be closed by means of a snugly fitting rubber stopper through the center of which passes a stout capillary tube of 2 mm. bore, extending 8 cm. beyond the rubber tube and ending in a beveled and tapered tip. This tube must fit the stopper tightly enough to resist being forced up or down in it. The entire unit fits into a metal holder which secures the stopper against the open end and permits the plunger to be forced down the length of the barrel by means of a screw-thread, forcing a fine cord of brain tissue from the capillary tip. To obtain a sample of brain, the syringe is immersed in the water bath and the plunger worked back and forth until it moves very freely. It is then pushed down just beyond the cutting edge of the barrel. This is now pressed against the brain and, with a slightly twisting motion, forced through the tissue, leaving the plunger free to move out as it is displaced by brain. When the cutting edge reaches the other side of the brain, it is closed by means of the rubber stopper and capillary tube. At this point pressure is applied against the plunger and some of the brain forced down the capillary to the tip. In this manner it is possible anaerobically to obtain a satisfactory 6 to 8 cc. sample of brain, representing both gray and white matter, sealed within a syringe.

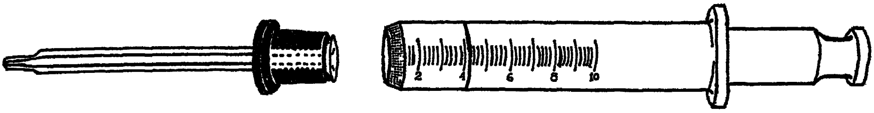

FIG. 1. The modified glass syringe used in obtaining samples of brain anaerobically.

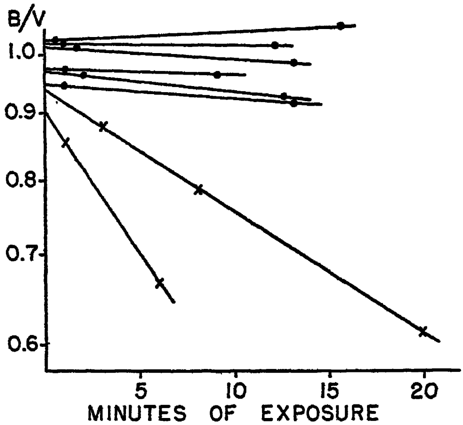

FIG. 2. The ratio of the nitrous oxide contents of brain and cerebral venous blood plotted semilogarithmically against the time of exposure of the brain before sampling. ×, samples obtained when the bath was equilibrated with 100 per cent O_2; ●, when equilibration was effected with the N_2O tension breathed by the animal.

The brain sampler was then removed from the bath, fastened into its holder, and an analysis for nitrous oxide carried out on 2 cc. samples by the analytical technique described above for blood and brain homogenate. Samples of torcular blood obtained at the time the animal was sacrificed were also analyzed for nitrous oxide by the same technique. The purpose of the water bath equilibrated with a tension of nitrous oxide equivalent to that inhaled by the animal is to minimize the loss of this gas from the brain in the brief time of exposure as the sample is being taken. This precaution was found to be necessary by preliminary experiments in which simply denitrogenated water was used as the bath (Fig. 2). It was found that there was a significant loss of nitrous oxide from the brain with time, if successive samples were taken after the dura was cut. When water equilibrated with 40 per cent nitrous oxide was used, however, it may be seen that this loss of nitrous oxide from the brain was prevented. It is worthy of note that in the earlier trials, in which loss occurred, if an exponential extrapolation is made to time of exposure = 0 the values obtained are comparable to those in which loss is actually prevented.

TABLE I

*Solubility of Nitrous Oxide in Dog and Human Whole Blood**

Dog		Man	
Animal No.	$\alpha^{37°}_{N_2O}$ (per cc. blood)	Red blood cell hematocrit	$\alpha^{37°}_{N_2O}$ (per cc. blood)
		per cent	
9	0.419	28.8	0.400
11	0.419	34.2	0.408
12	0.433	41.0	0.410
13	0.421	44.0	0.414
14	0.435	51.5	0.425
15	0.421		
Mean	0.425		0.412
S.E.	0.003		0.004

* $\alpha^{37°}_{N_2O}$ (per cc. of blood) = the number of cc. of N_2O (reduced to S.T.P.) dissolved by 1 cc. of blood when equilibrated at a nitrous oxide tension of 760 mm.

Results

Solubility of Nitrous Oxide in Blood and Brain in Vitro—The Bunsen solubility coefficient (Table I) for nitrous oxide in dog blood at 37° as determined by this technique was 0.425 (standard error = 0.003); this coefficient for human blood was 0.412 (standard error = 0.004). In the latter case a definite correlation may be noted between the proportion of red cells in the blood and the solubility of nitrous oxide in it. This was found to be the case for nitrogen by Van Slyke, Dillon, and Margaria (11). We found that the blood of a very anemic and a polycythemic individual varied from the mean by only 3 per cent. The values for α obtained by us in blood agree well with the value of 0.416 found by Orcutt and Seevers (12). The solubility of nitrous oxide in brain, however, has not hitherto been investigated. We found identical values for the whole brain of dog (0.437 ± 0.008) and of man (0.437 ± 0.005), the former studied immediately after sacrifice, the latter obtained in autopsy specimens 4 to 24 hours after death from a variety of diseases (Table II). From these values for blood and brain in both species a brain-blood partition coefficient of 1.03 and 1.06 may be calcu-

TABLE II

*Solubility of Nitrous Oxide in Dog and Human Whole Brain**

Dog		Man		
Animal No.	$\alpha^{37°}_{N_2O}$ (per gm. brain)	Patient	Condition	$\alpha^{37°}_{N_2O}$ (per gm. brain)
8	0.434	A. K.	Depressive psychosis	0.428
9	0.406	B. B.	Nephritis	0.434
11	0.430	S. F.	Apoplexy	0.437
12	0.420	M. Mc.	Cardiac failure	0.425
13	0.458	R. C.	Paresis	0.438
14	0.455	C. H.	Arteriosclerosis	0.432
15	0.454	J. J.	Hypertension	0.464
Mean.....	0.437			0.437
S.E.......	0.008			0.005

* $\alpha^{37°}_{N_2O}$ (per gm. of brain) = the number of cc. of N_2O (reduced to S.T.P.) dissolved by 1 gm. of brain when equilibrated at 37° at a nitrous oxide tension of 760 mm.

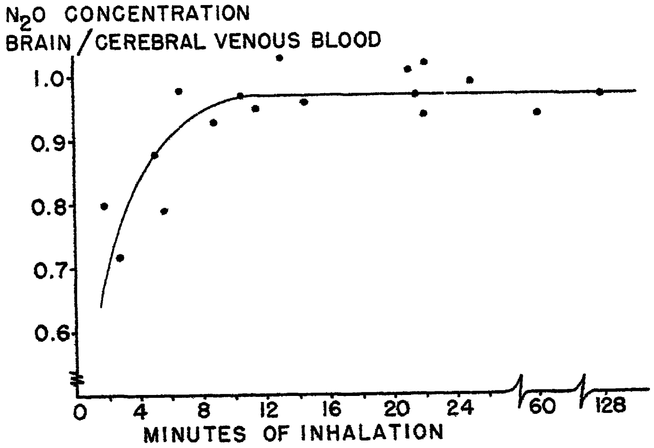

FIG. 3. The rate of equilibration of nitrous oxide between brain and cerebral venous blood *in vivo*.

lated for dog and man respectively, representing the ratio of the quantity of nitrous oxide dissolved in 1 gm. of brain to that in 1 cc. of blood when both are at the same tension.

Partition Coefficient in Vivo for Dog and Rate of Equilibration between Brain and Cerebral Venous Blood—In Fig. 3 are presented the data on the nitrous oxide content of brain and cerebral venous blood obtained simultaneously at times varying from 2 minutes to 2 hours after the onset of inhalation of the 40 per cent nitrous oxide mixture. It is seen that in the early period the brain has not come to equilibrium but that after about 10 minutes equilibrium is apparently established between brain and the blood draining the brain and that the ratio of the nitrous oxide content in the two phases remains unaltered up to 2 hours. The value for this brain-blood ratio in the five experiments with equilibration times from 8.6 to 13.3 minutes (0.970 ± 0.008) is not significantly different from that in the six experiments in which equilibration time was 20 minutes to 2 hours (0.975 ± 0.011). It is therefore possible to conclude that equilibration between brain and its venous drainage with respect to nitrous oxide tension is complete within 10 minutes, which is therefore the value of u in the equation for cerebral blood flow. The value for the brain-blood ratio of nitrous oxide concentrations at equilibrium (0.975) is the *in vivo* partition coefficient and agrees closely with the value for this constant obtained from *in vitro* equilibration (1.03 for the dog) (Table III).

TABLE III

Brain-Blood Partition Coefficient (S) *of Nitrous Oxide in Dog and Man*

Technique	Species	S	Standard error
In vitro................................	Dog	1.03	0.020
" "	Man	1.06	0.016
" *vivo*, 8–13 min. equilibration..............	Dog	0.970	0.008
" " 20 min. to 2 hrs. equilibration	"	0.975	0.011

DISCUSSION

An exact value for the solubility of nitrous oxide in the living human brain is of course not directly obtainable, although preliminary experiments in this laboratory have indicated that such values for radioactive gases are capable of direct estimation. Nitrous oxide solubility has, however, been measured in samples of human brain obtained at autopsy. We have demonstrated furthermore that this solubility in human brain tissue is identical with that in dog brain after death which, in turn, is remarkably close to the solubility in the living brain of that animal. It is, therefore, very likely that the same is true for man and that the solubility constant obtained *in vitro* is applicable to the living state. The experimentally determined values for the brain-blood partition coefficient of nitrous oxide lie on either side of and only slightly removed from unity and it seems fair to accept this value as a best approximation. The identity of the values found in dog and man speak for the dependence of this coefficient only on gross physicochemical constitution, which varies within extremely narrow limits despite major pathological changes (13–15). The small deviations found in the studies on brains from patients dying of a variety of diseases are further evidence for the constancy of this partition coefficient in different patients and divers pathological states. This value of unity for the partition coefficient is less than the value of 1.3 tentatively accepted on the basis of a few preliminary measurements (7). The numerous refinements in technique herein reported have undoubtedly yielded a more accurate evaluation of this constant.

In view of the high lipide content of brain tissue and the more than 3-fold greater solubility of nitrous oxide in common fats and oils over that in water or blood, it is surprising that the solubility of this gas in brain is not significantly greater than its blood solubility. In fact in two determinations on white matter (which has twice the lipide content of cortex) the nitrous oxide solubility was found to be within the range of that in whole brain (0.427 and 0.468). These findings are, however, not without precedent. In fact the majority of investigators who have studied, by somewhat cruder techniques, the contents in brain and blood of various volatile anesthetics have arrived at similar conclusions (2–6). Expecially noteworthy are the studies of Campbell and Hill (16) who found the nitrogen content of the whole brain at the usual atmospheric tensions to be 1.08 volumes per cent, not significantly different from the blood nitrogen content; yet these workers found the solubility of nitrogen in adipose tissue to be 5 times its solubility in blood. The conclusion is inescapable that brain lipides do not behave as do the usual fats and oils or even adipose tissue in their capacity for nitrous oxide and probably other gases. This does not necessarily vitiate the lipide theory of anesthesia, whose proponents may still reasonably ascribe a preferential absorption of volatile anesthetics to certain important lipides in the surface layers of nerve cells, even though the bulk of the lipides of central nervous tissue is indifferent to these gases.

The authors wish to acknowledge the cooperation of Dr. Helena E. Riggs, neuropathologist of the Philadelphia General Hospital, through whom the human brain samples were obtained.

SUMMARY

1. Techniques are described for the determination of the solubility of gases in brain *in vitro* and *in vivo*.

2. The Bunsen coefficient expressed as cc. of nitrous oxide (converted

to standard temperature and pressure) dissolved by 1 gm. of brain when equilibrated at a nitrous oxide tension of 760 mm. and 37° equals 0.437 ± 0.008 and 0.437 ± 0.005 for the mixed brain of dog and man respectively.

3. The brain-blood partition coefficient for nitrous oxide at 37°, expressed as the solubility per gm. of brain divided by the solubility per cc. of blood, was found to be 1.03 and 1.06 for dog and man *in vitro*, respectively, and 0.98 for the dog *in vivo*.

4. After approximately 10 minutes of inhalation of a constant tension of nitrous oxide there is complete equilibrium in the dog between brain and cerebral venous blood with respect to nitrous oxide tension.

5. The pertinence of these determinations to a method for measurement of cerebral blood flow in man and to the lipide theory of anesthesia is discussed.

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MICROBIOLOGICAL EVIDENCE FOR THE IDENTITY OF α- AND β-BIOTIN*

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Kögl (1, 2) has concluded that two forms of biotin exist in nature. The first of these, α-biotin, was isolated from egg yolk by Kögl and Tönnis (3), and the second, β-biotin, was isolated from liver and milk by du Vigneaud and associates (4–6), and more recently from liver by Kögl and ten Ham (1). Kögl based his conclusion on differences in the physical, chemical, and biological properties of the isolated compounds. There appears to be no doubt about the structure of the β-biotin, as this compound has been synthesized repeatedly and is now an article of commerce. While Kögl proposed a structural formula (7) for the so called α-biotin, he has not synthesized it. Melville (8), in his review, questions the conclusion that the two isolated biotins are different compounds.

One of the points of difference reported by Kögl was the response of yeast to the two compounds. Kögl and ten Ham (1, 2) reported that the activity of β-biotin was approximately twice that of α-biotin when tested by the "Rasse M" strain of yeast. Du Vigneaud and coworkers (4, 9) reported that a sample of α-biotin which they received from Professor Kögl had less than 50 per cent of the activity that their crystalline preparation from liver had for yeast, *Rhizobium trifolii*, and *Clostridium butylicum*. They pointed out, however, that this difference should not be stressed, since the Kögl sample might have suffered loss in potency, or might have been impure. Later revision of the melting point to a higher figure by Kögl and Pons (10) suggests that the earlier samples of α-biotin were not pure. In view of these differences and since we had a sample of Kögl's biotin, it seemed worth while to compare the activity of the two compounds for a number of microorganisms to see what light such tests might throw on the problem.

EXPERIMENTAL

Microbiological Assays—The organisms used were *Lactobacillus casei*, American Type Culture Collection, No. 7469, *L. pentosus* 124-2, *Saccharo-*

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myces cerevisiae Y-30, *Clostridium acetobutylicum* S-9, and *Neurospora crassa* 1-A wild.

The stock cultures of the lactobacilli were carried, inocula were prepared, and the procedure was carried out as described previously (11). The medium of Shull and Peterson (12) was used for *L. casei*. The medium for *L. pentosus* 124-2 contained, per liter, 10 gm. of glucose, 10 gm. of sodium acetate, acid-hydrolyzed casein equivalent to 5 gm. of casein, 0.1 gm. of L-cystine, 0.025 gm. of DL-tryptophan, 0.02 gm. of adenine sulfate, 0.25 mg. of riboflavin, 0.5 mg. of calcium pantothenate, 2.5 mg. of nicotinic acid, 0.25 mg. of *p*-aminobenzoic acid, and 5 ml. each of Salt Solutions A and B (11).

Saccharomyces cerevisiae was carried as a slant on the same medium as that for the lactobacilli. The inoculum was prepared by transferring from the slant to 10 ml. of the assay medium containing 1 mγ of biotin. After 18 to 24 hours growth the culture was centrifuged and the cells were resuspended in 10 ml. of sterile water. 1 drop of this suspension was used as inoculum for each flask.

The yeast assays were made in 50 ml. Erlenmeyer flasks. The medium contained, per liter, 10 gm. of glucose, 6 gm. of $NH_4H_2PO_4$, 1 gm. of KH_2PO_4, 1 gm. of asparagine, 1 gm. of sodium citrate, 0.25 gm. of $MgSO_4 \cdot 7H_2O$, 4 mg. of thiamine, 4 mg. of calcium pantothenate, 4 mg. of pyridoxine, 4 mg. of nicotinic acid, 100 mg. of inositol, and 1.0 ml. of the salt solution (0.2 gm. of $MnSO_4$, 0.2 gm. of $FeSO_4 \cdot 7H_2O$, and 0.1 gm. of $CuCl_2 \cdot 2H_2O$ per liter).[1] The final volume in the flasks was 10 ml. After inoculation the flasks were incubated at 30° on a reciprocating shaker making 90 strokes per minute which had a stroke distance of 10 cm. At the end of 16 hours the flasks were compared by reading light transmission in an 18 × 150 mm. tube in an Evelyn photoelectric colorimeter with a 660 mμ filter.

The inoculum for the *Clostridium acetobutylicum* assays was prepared by transfer from a soil spore stock to a 5 per cent corn mash medium. When this culture was actively gassing, usually after about 24 hours, transfer was made to a synthetic medium described in an earlier paper (13) containing 5 mγ of biotin. After 18 hours, another transfer was made to another tube of synthetic medium, this time containing only 1 mγ of biotin. This culture was used as an inoculum when it was actively gassing, usually after 12 to 18 hours, 1 drop to each tube.

The assays were conducted as in the method of Lampen and Peterson (13) for the assay of *p*-aminobenzoic acid, except that 0.1 per cent sodium thioglycolate was employed instead of reduced iron and sodium hydrosulfite. The tubes were read at 18 hours in an Evelyn photoelectric colorimeter with a 660 mμ filter.

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[1] Olson, B. H., unpublished data.

The inoculum for the *Neurospora crassa* assays was that described by Stokes *et al.* (14). Approximately 3 sq. mm. of spores from a slant consisting of 2 per cent agar, 0.2 per cent Difco yeast extract, and 0.2 per cent Difco malt extract, were suspended in 10 ml. of sterile water, and 1 drop of this suspension was used as the inoculum.

The assays were run in the medium of Horowitz and Beadle (15) with 10 ml. of medium in 50 ml. Erlenmeyer flasks. The mycelium was harvested after 5 days incubation at 30°. The pads were pressed out on filter paper, dried 12 hours at 90°, and weighed to the nearest 0.5 mg.

The sample of biotin methyl ester was received from Professor Kögl as a sterilized solution in 1940, and since that time has been kept in the refrigerator in a sealed ampule at a concentration of 1 γ per ml. in 50 per cent methyl alcohol. Since this sample was received before the question of the two forms of biotin had arisen (1, 2), it is presumably the α type. In his letter accompanying the sample, Professor Kögl stated that the biotin contained about 25 per cent impurity, and hence a 25 per cent correction has been applied in calculation of the amounts used. The methyl ester was hydrolyzed to the free acid before use. The β-biotin used was crystalline synthetic *dl*-biotin from Merck and Company, Inc. It has been shown (16) that only *d*-biotin has microbiological activity.

TABLE I

Comparative Activity of Kögl's Biotin and Synthetic dl-Biotin

Biotin per 10 ml.*	*Lactobacillus casei* 7469		*Lactobacillus pentosus* 124-2		*Saccharomyces cerevisiae* Y-30		*Clostridium acetobutylicum* S-9		*Neurospora crassa* 1-A wild	
	Acidity, ml. 0.1 N NaOH per 10 ml.				Evelyn readings, 660 mμ filter				Mg. mycelium per 10 ml.	
	M.†	K.†	M.	K.	M.	K.	M.	K.	M.	K.
mγ										
0	2.1	2.1	0.8	0.8	96	96	97	97	8	8
0.1	3.6	3.5	2.9	2.8	78	80	86	88	11	10
0.2	4.9	4.8	4.8	4.6	61	65	79	80	18	16.5
0.3					50	50			21.5	20.5
0.4	7.6	7.2	7.8	7.3						
0.5					34	35	70	70	31	29
0.6	10.1	9.9	9.5	9.3						
1.0			10.1	9.8					52	53
Comparative activity	100	90-96	100	94-95	100	90-94	100	90-93	100	90-96

* The Kögl sample was corrected for 25 per cent impurity; the amounts of synthetic *dl*-biotin represent only the *d* isomer.

† K., Kögl sample; M., Merck synthetic *dl*-biotin.

Results

Table I shows the comparative activities of the two biotins for five microorganisms. The activity of the Kögl biotin (which had been corrected for the 25 per cent impurity) was consistently from 90 to 96 per cent that of *d*-biotin (no activity for the *l* isomer being assumed). Du Vigneaud and coworkers (4, 9) have likewise reported the same *relative* activity for three microorganisms.

On the basis of these data two alternative assumptions may be made. The first is that the two compounds are identical, and hence have the same chemical and microbiological properties. The second is that the two biotins are chemically different, but elicit the same microbiological response. Some precedent for the latter view is found in the equal activity of biotin and oxybiotin for *Lactobacillus arabinosus* 17-5 (17–19) and *L. pentosus* 124-2.[2] However, oxybiotin is less potent than biotin for several other organisms, *e.g.* yeast (18–20) and *L. casei* (17–20). It is therefore hardly to be expected that, if the two biotins are not the same compound, they would have identical activities for five microorganisms as diverse as one anaerobic and two lactic acid bacteria, a yeast, and a mold. The microbiological data then lend support to the view that the two biotins are identical rather than different compounds.

SUMMARY

The activity ratio of Kögl's α-biotin to synthetic *dl*-biotin (β-biotin) for each of five microorganisms has been determined. In all cases Kögl's α-biotin was found to possess 90 to 96 per cent of the activity of the synthetic *dl*-biotin, assuming only *d*-biotin is active.

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THIAMINE AND THE CYANOGEN BROMIDE REACTION*

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Most chemical methods for the determination of nicotinic acid involve reaction with cyanogen bromide and an aromatic amine (1) or ammonia buffer (2). In some cases (3–10) heat is applied to the nicotinic acid-cyanogen bromide reaction mixture. During the course of investigations involving use of this reaction, it was found that thiamine, under certain conditions, also produces a colored compound with the reagent. The importance of this fact is appreciated when one considers that in general such determinations are carried out with the assumption that nicotinic acid is the only substance present which, with cyanogen bromide, will produce a colored compound measurable at the specific wave-length designated. Obviously, the presence of any other compound possessing this property could affect the accuracy of a nicotinic acid assay. Thiamine apparently is such a substance, since investigation has revealed that this vitamin, if present in relatively high concentration, does produce a colored compound with cyanogen bromide when the reaction is carried out at an elevated temperature.

EXPERIMENTAL

In studying the effect of thiamine on the cyanogen bromide reaction, the general procedure previously recorded for the determination of nicotinic acid (1) has been followed with respect to sample volume, cyanogen bromide reagent, buffer, and, when used, the amine reagent (*m*-phenylenediamine). In all colorimetric measurements a Klett-Summerson photoelectric colorimeter was employed. Since it was noted that addition of the aromatic amine was not required for the color to be produced by interaction of thiamine and cyanogen bromide, a study was made of the reaction without use of the amine. To accomplish this, 10 ml. samples of the thiamine solution plus 5 ml. of a buffer of pH 6.6 were heated on a steam plate for 10 minutes with 5 ml. of a 4 per cent aqueous solution of cyanogen bromide. Colorimetric readings (440 mμ) were made at varying times after removal from the steam plate. Table I presents the results so obtained. From these data it is apparent that the reaction between thiamine and cyanogen bromide is slow but that, given sufficient time, it is complete and the color developed is proportional to the amount of thiamine taken. The high concentration of thiamine required, however, makes the reaction impracticable as a general assay procedure for this vitamin.

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The production of color by thiamine in the presence of cyanogen bromide is not dependent upon the presence of an aromatic amine. Therefore, any cyanogen bromide-nicotinic acid assay procedure in which heat is employed might be subject to interference by the presence of a high concentration of thiamine. Such an effect is seen in Table II. In obtaining these data, the previous procedure (1), including use of *m*-phenylenediamine, was followed with the exception that the reaction mixture was heated (45–50°) for 5 minutes after addition of the cyanogen bromide and then placed at room temperature for 15 minutes before the aromatic amine was added. Heating was limited to 5 minutes, since further heating destroys the color produced by nicotinic acid, and since it is the maximum time employed in most procedures which include this step. The temperature was considerably lower than that used in methods requiring heat and serves to show that

TABLE I

Determinations with Pure Thiamine Solutions

Thiamine in sample	Density scale readings at varying times after removal from steam plate					
	15 min.	30 min.	45 min.	1 hr.	2 hrs.	3 hrs.
mg. per ml.						
0.00	0	0	0	0	0	0
0.05	46	50	52	55	57	57
0.10	70	80	82	90	105	110
0.15	105	120	125	135	158	165
0.20	140	158	166	180	210	220

TABLE II

Determination of Nicotinic Acid in Presence of Thiamine

50 γ of nicotinic acid in 10 ml. of sample.

Thiamine added	Density scale reading	Apparent recovery of nicotinic acid	
mg. per ml.		γ	*per cent*
0.00	130		
0.00	130		
0.05	150	57.7	115.4
0.10	175	67.3	134.6
0.15	200	76.9	153.8
0.20	222	85.4	170.8

for color production with thiamine the heat treatment does not need to be extreme. It is obvious from the data of Table II that under these conditions a nicotinic acid assay, in the presence of a high thiamine concentration, would be subject to error. Apparently, however, the reaction with thiamine is so slow that no error is involved in determinations in which the cyanogen bromide reaction is carried out at room temperature.

SUMMARY

Thiamine, under certain conditions, produces with cyanogen bromide a colored compound measurable at the wave-lengths commonly employed for the quantitative determination of nicotinic acid. Since production of this colored compound results when the thiamine concentration is relatively high and when the cyanogen bromide reaction is carried out at an elevated temperature, a potential analytical hazard exists in those nicotinic acid assay procedures in which heat is applied to the reaction mixture.

Although interference by nicotinic acid can easily be overcome by prolonging the time of heating, utilization of this reaction for the quantitative determination of thiamine does not appear to be generally practicable because of the high concentration of thiamine required.

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A PHOTOMETRIC METHOD FOR THE DETERMINATION OF FREE PENTOSES IN ANIMAL TISSUES*

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Most of the micromethods for the determination of pentoses in tissues are based upon the formation of furfural and the reaction of the latter compound with such reagents as aniline (1), benzidine (2), xylidine (3), or orcinol (4). The furfural is formed in these methods usually by heating the unknown in a fairly concentrated solution of strong mineral acid at temperatures ranging from 100–175°. The drastic conditions used in these methods produce furfural not only from pentoses and pentosans but also from other carbohydrates, glucuronic acid (1), and ascorbic acid (5). Methods involving such severe conditions are thus highly non-specific for free pentoses in tissue extracts.

We have developed a method for the determination of free pentose in animal tissues after the administration of pentose. The basis of our procedure is the reaction of *p*-bromoaniline acetate with furfural. The reaction is carried out at 70°, an adaptation that obtains a high degree of specificity.

Acetic acid in a concentration of approximately 83 per cent was found to be more favorable for the conditions of our procedure than strong mineral acids. It was observed that an interfering brown color is produced when an acetic acid solution of aniline mixed with tissue filtrate is heated. This brown color is due to the presence of oxidation products of aniline. A study of aniline derivatives showed that the *p*-bromo-substituted compound is more resistant to the formation of the interfering colored products than is aniline; hence the use of *p*-bromoaniline was adopted.

Another improvement of great importance was the addition of an antioxidant, thiourea, to the color-producing reagent. The reagent finally developed consists of 2 per cent *p*-bromoaniline in glacial acetic acid saturated with thiourea.

The problem of preparing tissue extracts not containing objectionable amounts of interfering substances was solved by using Somogyi's deproteinizing reagents (6), barium hydroxide and zinc sulfate. The Somogyi reagents yield filtrates essentially free from combined pentose compounds from which small amounts of furfural would be formed by heating with our color-producing reagent. These filtrates, however, contain some interfering substances, such as glucuronic acid and glucose. Interference from such substances is avoided by preparing a tissue extract of 1:20 dilution or greater. With 1:20 filtrates of muscle, brain, kidney, spleen, blood, and hide of the fasted rabbit, practically no color is obtained by our procedure. However, with liver filtrates of a 1:20 dilution a color is obtained which amounts to about 6 mg. of apparent pentose per 100 gm. of tissue. Glycogen in liver filtrates does not yield interfering color but produces turbidity. This objection is not serious, as experimental work is usually carried out on fasted animals.

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TABLE I

Interference Observed with Authors' Method and with Orcinol Reagent at 70°

Compound	Color produced by 0.1 mg. D-ribose per cc.			
	Authors' method, mg. per cc.		Orcinol reagent at 70°, mg. per cc.	
	0.1	1.0	0.1	1.0
	per cent	*per cent*	*per cent*	*per cent*
Glucose	1.0	8.5	5.1	8.9
Fructose	0.0	0.0	7.1	127.0*
Ascorbic acid	0.0	0.0	6.5	42.3
Dehydroascorbic acid	0.0	2.1	0.8	34.2
Na glucuronate	0.0	2.4	5.4	53.5
Galactose	4.0	21.6	1.6	5.3
Sucrose	0.5	1.0	3.9	43.5
Maltose	0.0	0.0	6.5	8.3
Lactose	0.0	1.8	2.5	3.3
Soluble starch	0.0	0.0	5.1	5.7
Glycogen	0.0	0.8	27.6	32.2
Gum arabic	5.2	22.2	39.5	114.0

* Solution became turbid.

We found the orcinol reaction of Bial less satisfactory for the determination of pentoses than the reaction with *p*-bromoaniline as developed by us. When the orcinol reagent is mixed with test material and boiled at 100° for 5 to 20 minutes, as recommended by different authors (4, 7), the interference from non-pentose material is very great. Even under the mild conditions of our procedure the orcinol reagent gives considerable interference from non-pentose substances, as shown in Table I. The data of Table I were obtained by mixing 1 cc. of unknown solution with 5 cc. of reagent, warming in a water bath at 70° for 10 minutes, and cooling promptly to room temperature. The orcinol reagent was essentially the same as that used by Mejbaum (4), Militzer (7), and others. It consisted of a mixture of 1 volume of 1 per cent aqueous orcinol, 0.1 volume of 10 per cent $FeCl_3$ solution, and 4 volumes of concentrated HCl, freshly prepared. The data show that greater interference results with the modified orcinol method than with the authors' procedure.

Technique of New Method

Reagents—

1. p-Bromoaniline reagent. Thiourea is added to glacial acetic acid in excess of the amount that will dissolve. Approximately 4 gm. of thiourea per 100 cc. of acetic acid are used. Decant 100 cc. of acetic acid saturated with thiourea and dissolve 2 gm. of pure *p*-bromoaniline (Eastman Kodak Company) in it. Keep the *p*-bromoaniline reagent in a dark glass bottle and prepare about once weekly.

2. Somogyi deproteinizing reagents (6). 5 per cent solution of $ZnSO_4 \cdot 7H_2O$ and 0.3 N barium hydroxide solution are prepared. These reagents must neutralize each other precisely, volume for volume, when titration is performed with phenolphthalein as indicator.

3. Sugar standard. A stock solution of pentose is made up by dissolving reagent grade sugar in saturated benzoic acid solution. A working standard containing 0.1 mg. per cc. is prepared by diluting the stock solution with saturated benzoic acid.

Procedure

The filtrate of the tissue is made with an expected concentration of 0.01 to 0.2 mg. per cc. The following procedure is used for a 1:20 dilution: Place in a Waring blendor, or a mortar, a convenient quantity of tissue. Add 15 volumes of water and homogenize until a thoroughly dispersed mixture is obtained. Add 2 volumes of 0.3 N barium hydroxide and mix thoroughly. Now add without delay 2 volumes of 5 per cent $ZnSO_4 \cdot 7H_2O$ and mix thoroughly. Filter.

Place 1 cc. of filtrate in each of two photoelectric colorimeter tubes. In each of two similar tubes place 1 cc. of standard pentose solution containing 0.1 mg. per cc. To each of the tubes add 5 cc. of the *p*-bromoaniline reagent and mix thoroughly. Place one tube containing the standard solution and one tube with the unknown filtrate in a water bath at 70°, keeping a standard and an unknown tube to serve as unheated blanks. Keep the tubes in the water bath for 10 minutes, then remove, and cool in running water until the tubes have reached room temperature. Set the tubes in the dark for 70 minutes, then read in a photoelectric colorimeter, using a 520 mμ filter. The unheated tubes are used as blanks for adjusting the colorimeter. Take the readings and calculate the unknown in terms of the pentose standard used.

TABLE II

Agreement with Beer's Law

	Photometric density, $L = 2 - \log G$		
	D-Arabinose	D-Ribose	D-Xylose
γ per cc.			
100	0.3660	0.3980	0.4410
50	0.1821	0.2007	0.2255
25	0.0908	0.1051	0.1142
12.5	0.0452	0.0537	0.0555
6.25	0.0218	0.0252	0.0298

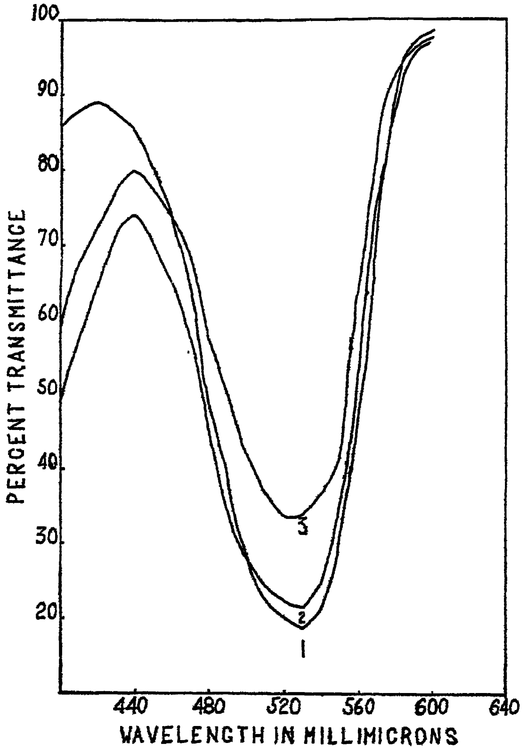

FIG. 1. Absorption curves of the chromogen formed by interaction of *p*-bromoaniline reagent with the following: Curve 1, furfural; Curve 2, D-xylose; Curve 3, D-arabinose. A Coleman spectrophotometer, model 10-S, was used.

DISCUSSION

The proportionality of color obtained in this method is in excellent agreement with Beer's law. This is shown by the data of Table II for D-arabinose, D-ribose, and D-xylose. Readings were made with the Evelyn photoelectric colorimeter.

The color obtained in this procedure reaches a maximum in approximately 60 minutes and remains constant for about 30 minutes at temperatures of 20–25°, then fades slowly. It is more stable at lower temperatures. We found it to keep constant in intensity for 48 hours in a refrigerator at 8°. This color fades slowly upon exposure to light.

In view of the effect of temperature and light upon the intensity and stability of this color, we recommend that a standard solution of pentose be used for color comparison instead of attempting to standardize conditions so that calibration curves may be made. The use of a single standard is satisfactory because of the excellent agreement of the color intensity with Beer's law.

TABLE III

Experiments Showing Recovery of Pentoses Added to Animal Tissues

Tissue	Sugar	Amount added	Recovery
		mg. per gm.	*per cent*
Liver	D-Arabinose	1.0	93.6
Kidney	"	1.0	98.2
"	"	0.02	96.0
Muscle	"	0.02	101.5
"	D-Xylose	1.0	99.3
Brain	D-Arabinose	2.0	101.8
"	D-Xylose	0.02	94.5
Blood	D-Arabinose	1.0	98.5
"	"	0.02	106.5
Intestine and contents	"	1.0	101.8
" " "	"	0.02	101.3
Urine	"	0.02	103.8

The absorption curves of Fig. 1 offer good proof that the color obtained in the procedure reported is due to the formation of furfural and the reaction of the latter with *p*-bromoaniline acetate.

The recoveries obtained when pentoses were added to animal tissues are shown in Table III.

In this procedure only about 9 per cent of the available furfural appears to be liberated in 10 minutes at 70°. Approximately 4 times as intense a color is produced by heating at 100° for 5 minutes. One may therefore make this procedure more sensitive by heating the reaction mixture to a higher temperature. This is inadvisable, however, except in analyses of materials free from other furfural precursors.

This method has been successfully used in studies of the metabolic fate of D-arabinose, L-arabinose, and D-xylose in the rabbit which will be reported later.

SUMMARY

A method for the determination of free pentoses in animal tissues has been developed. The method is based upon the formation of furfural from pentose in 83 per cent acetic acid containing thiourea at 70° and the reaction of the furfural with *p*-bromoaniline acetate to form a pink-colored product.

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A STUDY OF THE CONVERSION OF ISOTOPIC NICOTINIC ACID TO N^1-METHYLNICOTINAMIDE

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(Received for publication, December 15, 1947)

On the basis of fluorometric estimations, N^1-methylnicotinamide chloride (NMN) has been reported to be one of the principal urinary excretion products of nicotinic acid in man (1–5), dogs (6), pigs (7), rats (8, 9), and cotton-rats (10). Huff and Perlzweig (2) have isolated this substance from urine after feeding large doses of nicotinamide to man.

In addition it has been reported that the ingestion of tryptophan results in an increased excretion of NMN (9, 11–13), indicating that this compound may have at least two dietary precursors.

As a preliminary to other experiments dealing with the conversion of tryptophan to NMN, it was necessary to prove the conversion of nicotinic acid to NMN. This has been done by using an isotope tracer technique. Our results indicate that nearly all of the urinary NMN comes from the ingested nicotinic acid under the conditions employed.

EXPERIMENTAL

Synthesis of Labeled Nicotinic Acid—Nicotinic acid[1] containing C^{13} in the carboxyl position was synthesized[2] according to the method of Murray, Foreman, and Langham (14). The final product melted at 227.5–229° (uncorrected) and contained 0.81 per cent excess C^{13}, corresponding to an excess of 4.86 per cent C^{13} in the carboxyl group. Commercial nicotinic acid (Merck) was used as the control material.

Feeding of Labeled Nicotinic Acid—Eight male rats, weighing an average of 339 gm. each, had been maintained for several months on a nicotinic acid-free diet consisting of casein 12, L-cystine 0.15, sucrose 81, cottonseed oil 3, salts 4 (15), and the usual vitamins (16). On this diet rats excrete a negligible amount of NMN (16).

1 gm. of the labeled nicotinic acid was added to 1 kilo of the above ration and fed until completely consumed. Urines were collected daily under toluene and kept at 4° during the 14 day collection period.

Other Methods—Estimations of NMN were made by the method of Huff and Perlzweig (17). Spectrophotometric determinations[3] were made on a Beckman spectrophotometer. All isotope determinations were made on the Consolidated mass spectrometer of the National Bureau of Standards.[4] Samples were prepared for analysis by chromic-sulfuric acid oxidation. NMN was extracted and purified from the urine according to the method of Huff and Perlzweig (2) with certain modifications as indicated.

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[1] The C^{13} was donated through the courtesy of Dr. Sidney Weinhouse, Houdry Process Corporation, Marcus Hook, Pennsylvania.

[2] Kindly synthesized for us by Dr. Wright Langham, Los Alamos Scientific Laboratory, Santa Fe, New Mexico.

Results

5450 ml. of urine (including funnel washings) contained 681 mg. of NMN as determined by fluorometric assay, equivalent to a 48.6 per cent return of the ingested nicotinic acid.

The NMN was extracted as recommended by Huff and Perlzweig except that absolute methanol was used in place of 95 per cent ethanol, and 25 per cent sodium chloride was used for the permutit elutions. After the eluate had been freed of sodium chloride, assay indicated that it contained 286 mg. of NMN. The picrate was formed in 95 per cent ethanol and allowed to crystallize in the cold. After removal of the free picric acid the crystals were separated into two portions by fractional crystallization from 95 per cent ethanol. One fraction was only sparingly soluble and consisted of small irregularly shaped crystals which became suspended very readily in the alcohol. These crystals melted at 227° (uncorrected) and have not been completely identified. The second fraction dissolved more readily in boiling alcohol and crystallized in flat yellow leaflets, or when crystallized from more dilute solutions in short prismatic bars. The melting point after repeated recrystallizations was 189–189.5° (uncorrected) and showed no depression when mixed with NMN picrate prepared from synthetic NMN,[5] the melting point of which was 189.5° (uncorrected). The melting point of the picrate of nicotinic acid (recrystallized one time) was 217° (uncorrected), indicating that the isolated picrate contained little, if any, nicotinic acid.

When rats, diet, and extraction procedures were employed as described above, NMN picrate was isolated after feeding normal nicotinic acid for comparison with the tagged samples and to guard against any natural isotope shift. A picrate having the same crystalline structure and melting point as the tagged compound was obtained.

Aliquots of the various purified picrate samples were then treated to obtain the free NMN. The procedure used by Huff and Perlzweig (2) was followed except that ether saturated with water was used instead of absolute ether to minimize loss in the water phase. When crystallized from a minimum amount of boiling absolute ethanol, typical rosettes were obtained. When crystallized from more dilute solutions, the crystals appeared as prismatic bars.

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[3] We wish to thank Dr. George A. Hottle for performing these determinations.

[4] We wish to thank Dr. Fred Mohler and his staff for doing these analyses for us.

[5] W. A. Taylor and Company, Inc., Baltimore, Maryland.

The free compounds were examined spectrophotometrically, all showing identical absorption peaks at 2650 A with no evidence of a maximum at 2600 A characteristic of nicotinamide. Further, when the compounds were compared with one another and to synthetic NMN as a standard, each sample appeared to be 100 per cent pure within the limits of error of weighing the 2 mg. samples used. Fluorometric assays of the various samples also indicated approximately 100 per cent purity as compared to synthetic NMN.

Melting points and isotope analyses are indicated in Table I. It is evident from the isotope analyses that the concentration of C^{13} in the NMN isolated from urine was almost as great as in the nicotinic acid fed. When allowance was made for the fact that NMN contains 1 more carbon atom than does nicotinic acid, the tagged NMN had 95.7 ± 0.7 per cent of the original C^{13} concentration, a dilution of only 4.3 per cent.

TABLE I

Isotope Analyses and Melting Points of Free Compounds Obtained from Picrates

N^1-Methylnicotinamide	M.p. (uncorrected)	Temperature of decomposition	Excess C^{13}*
	°C.	°C.	*per cent*
Tagged	230–230.5†	233	+0.664‡ ± 0.005
Normal	233	233.5	+0.013 ± 0.003
Synthetic§	230–230.5	233	−0.002 ± 0.003

* CO_2 from dry ice served as the standard for all analyses. The figures given are the average of at least twelve repeated measurements, the plus-minus figures representing the average deviation from the mean.

† Huff and Perlzweig (2) give 233–234° (uncorrected) as the melting point of NMN.

‡ This corresponds to 4.64 per cent excess C^{13} in the carboxyl group as compared to the original 4.86 per cent excess C^{13}.

§ Obtained from the picrate of commercial NMN with the same procedure as was used with the tagged and normal samples.

No estimates of total yield from the extraction procedures were made, since only the purest portions of the picrate fractions were subsequently converted to the free NMN.

DISCUSSION

It seems clear from the data reported that nicotinic acid is a precursor for urinary NMN. Since only 4.3 per cent of the NMN was not tagged, it is evident that very little came from sources other than the ingested nicotinic acid under the conditions of this experiment.

The 4.3 per cent normal NMN found could have come from two sources: (1) the nicotinic acid and related compounds present in the rats at the start of the feeding period and (2) NMN synthesized from tryptophan or other sources during the feeding period.

Since the whole carcass content of nicotinic acid has been determined in rats (18), it was estimated that these rats contained a maximum of 89 mg. of nicotinic acid[6] and related compounds. Assuming that the body stores of nicotinic acid were completely replaced by the ingested nicotinic acid, and assuming further that the same percentage of this nicotinic acid was recovered in the form of NMN as the observed percentage of recovery from the ingested nicotinic acid (48 per cent), one would expect 59.8 mg. of the 681 mg. of NMN (8.8 per cent) originally present in the urine to be untagged. This is more than sufficient to account for the observed dilution (4.3 per cent).

Hence it seems likely that there was no appreciable synthesis of nicotinic acid from tryptophan or other sources in this experiment. However, since the level of nicotinic acid fed was at least 50 times the physiologically required amount on this diet (16), it might be expected that nicotinic acid synthesis from sources such as tryptophan would have been inhibited perhaps following the law of mass action.

These data have not been interpreted to indicate that rats cannot synthesize nicotinic acid from tryptophan. There is abundant evidence on a non-isotope basis that they can do so under proper circumstances (9, 11, 16).

SUMMARY

Rats fed large amounts of carboxyl-labeled nicotinic acid excreted N^1-methylnicotinamide chloride which contained 95.7 per cent of the original concentration of C^{13}, indicating that nicotinic acid is a precursor for N^1-methylnicotinamide.

Under the conditions of this experiment, rats excreted little, if any, N^1-methylnicotinamide from sources other than the ingested nicotinic acid and the body stores.

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[6] Actually these rats probably contained only about two-thirds of this amount, since the diet used produces a mild nicotinic acid deficiency. Repeated assays of muscle and liver from rats on this diet have shown about two-thirds of the "normal" amount of nicotinic acid. Whole carcass determinations have not been made however.

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INHIBITION OF COUPLED PHOSPHORYLATION IN BRAIN HOMOGENATES BY FERROUS SULFATE*

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Previous studies have shown a marked effect of purified preparations of certain neurotropic viruses and of iron salts on glycolysis of mouse brain homogenates. Colorimetric determinations of the iron content of purified and well dialyzed preparations of the Theiler FA strain of mouse encephalomyelitis virus gave values for iron corresponding to the inhibitory action of these virus preparations on glycolysis (1).

Experiments with intermediary metabolites of glucose breakdown pointed to an impairment of the phosphorylating steps leading to the formation of fructose-1,6-diphosphate. The addition of this latter compound to brain homogenate inactivated by ferrous sulfate resulted in a rapid rate of lactic acid production. However, it was not possible to restore glycolytic activity to the inactivated homogenate by adding an excess of the enzymes hexokinase and phosphofructokinase which are known to catalyze the formation of fructose-1,6-diphosphate. A fraction prepared from rabbit muscle ("restoring factor") which had no hexokinase or phosphofructokinase activity fully restored the activity to the iron-inactivated homogenate as well as to homogenates of mouse brains infected with the Theiler FA strain of mouse encephalomyelitis virus.

It is the purpose of this paper to report evidence which indicates that the restoring factor is identical with glyceraldehyde phosphate dehydrogenase (triose phosphate dehydrogenase). The effect of the partial inactivation of this enzyme in the glycolyzing brain homogenates by iron salts is most apparent in the phosphorylation reactions because the energy-rich phosphate which is derived from the oxidation of 3-phosphoglyceraldehyde becomes the limiting factor in the formation of fructose-1,6-diphosphate. It will be shown that some inactivation of phosphofructokinase also occurs in the presence of iron salts after prolonged incubation at 37°.

Finally, a study was made of the factors in brain homogenates which, in the presence of iron salts, inactivate these two glycolytic enzymes.

Methods

The methods used for the preparation of brain homogenate and of coenzymes were described previously (1). Phosphocreatine was synthesized

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as the calcium salt (2) and converted into the potassium salt before use. Glucose determinations were made according to the method of Nelson (3). Triose phosphate dehydrogenase was determined by the spectrophotometric test described by Warburg and Christian (4).

Results

Effect of Fluoride and Arsenate on Inhibition Produced by Ferrous Sulfate—Instead of determining glycolytic activity by lactic acid production as was usually done in previous studies, glucose disappearance was followed by chemical determination of glucose after deproteinization by $Ba(OH)_2$-$ZnSO_4$ (3). Thus it was possible to analyze effects of inhibitors with known modes of action. Potassium fluoride was used to inhibit enolase and potassium arsenate was added to eliminate the energy-rich phosphate produced by the oxidation of phosphoglyceraldehyde (4). Despite addition of an excess of phosphocreatine, optimal phosphorylation of glucose was not obtained in the absence of DPN (diphosphopyridine nucleotide). Moreover, ferrous sulfate had little inhibitory effect under these conditions (Table I). When DPN was added, glucose phosphorylation was doubled in the normal brain homogenate but was without effect in the iron-inacti-

TABLE I

Effect of Potassium Arsenate, Potassium Fluoride, and DPN on Glucose Phosphorylation in Normal and Iron-Inactivated Brain Homogenates

0.3 ml. of brain homogenate in 0.01 M potassium phosphate, pH 7.4; 0.4 ml. of 0.03 M glucose and 0.1 ml. of a solution containing 50 γ of ferrous sulfate·$7H_2O$ per ml. (0.1 ml. of H_2O in the controls) incubated for 20 minutes at 38°; then were added 0.1 ml. of 0.16 M $KHCO_3$, 0.1 ml. of 0.1 M ammonium phosphate, pH 7.6, 0.1 ml. of 0.15 M potassium arsenate,* 0.1 ml. of 0.07 M $MgCl_2$, 0.1 ml. of 0.01 M ATP, 0.5 ml. of 1 per cent phosphocreatine, 0.1 ml. of 4 per cent nicotinic acid amide (NAA),* 0.1 ml. of 0.7 per cent DPN,* and 0.1 ml. of 0.6 M potassium fluoride;* final volume used 2 ml. Incubation, 30 minutes at 38°. The determinations of glucose were made on the $Ba(OH)_2$ and $ZnSO_4$ filtrate.

Additions	Glucose disappearance after 30 min.		
	Normal brain homogenates	Iron-inactivated brain homogenates	Inhibition
	micromoles	*micromoles*	*per cent*
None	3.6	2.9	19†
KF	4.8	4.3	10†
NAA + DPN + KF	7.3	4.3	41
" + "	8.0	3.8	53
Arsenate + NAA + DPN + KF	4.0	3.9	3

* Added only when indicated.

† Small amounts of DPN were present in the freshly prepared brain homogenate.

vated brain. This lack of DPN effect in the inactivated brain was observed, both in the presence and absence of potassium fluoride. Since potassium fluoride blocks the formation of phosphopyruvic acid from phosphoglyceric acid, the only energy-rich phosphate which can be obtained under such experimental conditions is derived from the oxidation of phosphoglyceraldehyde to phosphoglyceric acid. This reaction is catalyzed by the phosphoglyceraldehyde dehydrogenase (oxidizing enzyme) and requires the presence of DPN and inorganic phosphate.

Upon addition of arsenate which can replace inorganic phosphate in this reaction, the production of energy-rich phosphate is avoided, without interference with the oxidation of phosphoglyceraldehyde. It can be seen from Table I that in the presence of potassium arsenate and potassium fluoride iron salts have no inhibitory effect even when DPN is present.

TABLE II

Stimulation of Glucose Phosphorylation in Iron-Inactivated Brain Homogenates by Fructose-1,6-diphosphate

Brain homogenate and solutions as in Table I. No addition of phosphocreatine, potassium fluoride, or arsenate. Preliminary incubation with ferrous sulfate and glucose for 20 minutes at 38°; then for 60 minutes at 38° after addition of $MgCl_2$, ATP, DPN, NAA, and $KHCO_3$.

Brain preparation	Addition	Glucose disappearance
		micromoles
Normal mouse brain homogenate		13.8
Same + 5 γ ferrous sulfate		2.1
" + 5 " " "	Fructose-1,6-diphosphate, 2 micromoles	6.4

These experiments strongly point, therefore, to the glyceraldehyde phosphate dehydrogenase as the locus of inhibition by iron salts.

Restoration of Glucose Phosphorylation to Iron-Inactivated Brain Homogenates—Inhibition of the triose phosphate-oxidizing enzyme was, however, in apparent contradiction to previously obtained results showing rapid lactic acid production from fructose-1,6-disphosphate in brains infected with the Theiler FA virus or inactivated by iron salts.

The large amounts of fructose-1,6-diphosphate which were added in these earlier experiments cannot be compared to the small quantities present in the course of glucose phosphorylation. It was necessary, therefore, to test the effect of small amounts of fructose-1,6-diphosphate on the disappearance of glucose. It was found (Table II) that 2 micromoles of fructose-1,6-diphosphate caused the disappearance of 4 to 5 micromoles of glucose in the homogenate, in addition to the 2 micromoles which are phosphorylated in the absence of fructose-1,6-diphosphate. This effect of fructose-1,6-diphosphate on glucose phosphorylation confirmed on the one hand the utilization of this substance in the inactivated brain and stressed on the other hand the impairment of the phosphorylation mechanism in the presence of iron salts.

These three observations, namely the utilization of fructose-1,6-diphosphate, the impairment in the activity of the triose phosphate-oxidizing enzyme, and the capacity of hexose diphosphate to stimulate glucose phosphorylation in the inactivated brain homogenate, led to the following working hypothesis of the mechanism of the iron salt or virus inhibition.

Production of energy-rich phosphate is the limiting reaction in brain homogenates. A partial inactivation of the coupled phosphorylation by the triose phosphate-oxidizing enzyme leads to a marked depression of the energy-rich phosphate supply which, in turn, results in impaired production of hexose diphosphate. If the oxidation of glyceraldehyde phosphate is not the limiting factor, such a vicious circle could block effectively phosphorylation of glucose without noticeably impairing lactic acid production from fructose-1,6-diphosphate.

To test this hypothesis the following experimental approach was used: (*a*) The triose phosphate-oxidizing enzyme was prepared from yeast and from rabbit muscle, and during the course of purification parallel determinations of enzyme activity and measurements of restoring capacity were made. (*b*) The final product of purification from rabbit muscle which was recrystallized several times was tested for restoring capacity and for inactivation by the brain homogenates in the presence of iron. (*c*) Attempts were made to restore to a large extent glucose phosphorylation by a supply of energy-rich phosphate from a source other than oxidation of glyceraldehyde phosphate.

Purification of Glyceraldehyde Phosphate Dehydrogenase from Yeast and Muscle—Purification of the phosphoglyceraldehyde-oxidizing enzyme of yeast was carried out according to the description by Warburg and Christian (4). All the fractions of the purification procedure were tested for glyceraldehyde phosphate dehydrogenase activity spectrophotometrically, and for presence of restoring factor by adding them to brain homogenates inactivated by ferrous sulfate.

The details of the purification procedure need not be repeated. The following observations were made with the use of Fleischmann's (bakers') yeast which was dried in thin layers for 5 days at room temperature.

Approximately 60 per cent of the total activity of the phosphoglyceraldehyde dehydrogenase is precipitated with 35 volumes per cent of acetone. By further addition of acetone to 55 volumes per cent, one-fourth of the total activity can be recovered. For further purifications only the first acetone precipitate was used.

The isoelectric precipitates were all collected and tested because of observations made by Meyerhof and Junowicz-Kocholaty (5) with American bakers' yeast that a departure from the original method was required. In our experience with Fleischmann's (bakers') yeast, the purification procedure of Warburg and Christian (4) can be followed without modification. Most of the activity remained in the supernatant at pH 4.5 and was precipitated upon addition of nucleic acid. The nucleic acid was removed with protamine sulfate (Squibb).

TABLE III

Comparison of Glyceraldehyde Phosphate Dehydrogenase and Restoring Activity in Fractions from Bakers' Yeast Maceration Juice

Determination of glyceraldehyde phosphate dehydrogenase activity in the Beckman spectrophotometer (4). Units are expressed as the change in density for the 1st minute multiplied by 1000. 1 unit of restoring activity is expressed as the capacity to bring about 50 per cent restoration to an iron-inactivated brain homogenate.

	Glyceraldehyde phosphate dehydrogenase		Restoring factor	
	Units per ml.	Specific activity	Units per ml.	Specific activity
1st extract	150,000	2,400	900	14
35% acetone ppt.	260,000	6,000	600	15
50% " "	112,000	4,300	350	13
pH 4.5 ppt.	200,000	8,000		
" 4.5 supernatant	42,000	5,000	120	14
Nucleic acid ppt.	560,000	8,600	1500	23
" " supernatant	13,000	4,300	30	10
After heating*	260,000	30,000	450	54

* The heating procedure was applied to the nucleic acid precipitate after removal of the nucleic acid by protamine sulfate.

The restoring activity of the yeast fractions was measured by determining the amount required to give 50 per cent restoration to a brain homogenate inactivated under standardized conditions. A linear relationship between per cent restoration and amounts added was established only for a narrow range in the neighborhood of 50 per cent restoration. The activity determination lacked accuracy unless measured in this range. From Table III it is apparent that fairly good agreement exists between the phosphoglyceraldehyde dehydrogenase activity and the capacity to restore glycolysis to the iron-inactivated brain homogenate in all the fractions except for the first crude extract, which showed a relatively higher restoring capacity.

Phosphoglyceraldehyde dehydrogenase has been crystallized from rabbit muscle by Cori *et al.* (6) by a simple and reproducible method. The product obtained by this method was recrystallized six times and tested for restoring activity after it had been well dialyzed and then diluted in the presence of cysteine. It was found to be very active in restoring glycolytic activity to the iron-inactivated brain.

During the purification procedure a comparative study of the relative triose phosphate dehydrogenase and restoring activity in fractions obtained from rabbit muscle was not made because of the following two difficulties encountered with the muscle preparations: (*a*) The spectrophotometric determination of the triose phosphate-oxidizing enzyme is not feasible with the crude extracts because of the presence of large quantities of α-glycerophosphate dehydrogenase which interfere with activity measurements. (*b*) A concentration of 0.02 M ammonium sulfate inhibits glycolysis almost completely, indicating a high sensitivity of the brain homogenate to this salt. This difficulty was not fully appreciated in earlier experiments.

Effect of Brain Homogenate and Iron Salts on Added Purified Triose Phosphate-Oxidizing Enzyme—When a preparation of a six times recrystallized glyceraldehyde phosphate dehydrogenase from rabbit muscle is added to a brain homogenate, it is rapidly inactivated if iron salts are present. Some inactivation, although at a slower rate, occurs in the absence of iron salts. Large quantities of the triose phosphate enzyme had to be added to allow for subsequent dilution in order to avoid interference by the brain homogenate in the spectrophotometric test. In a typical experiment 0.1 ml. of 0.24 M glucose, 5 γ of ferrous sulfate, and a six times recrystallized preparation of glyceraldehyde phosphate dehydrogenase from rabbit muscle were added to 0.3 ml. of a freshly prepared brain homogenate and the mixture incubated at 38° for 45 minutes. For the spectrophotometric test, 0.05 ml. of a 1:100 dilution of the mixture in a cysteine-pyrophosphate buffer of pH 8.0 was used. The activity of the diluted sample at zero time was 1000 units per ml. (with an experimental error of about 10 per cent). After 45 minutes the 1:100 dilution of the iron-inactivated brain homogenate contained only 300 to 500 units, representing 50 to 70 per cent inactivation, while the activity of the mixture containing normal brain homogenate was depressed by 20 to 30 per cent. These findings are in good agreement with the observations on the over-all glycolytic activity which also falls off slowly in the absence of added ferrous sulfate when subjected to prolonged incubation at 38°.

Restoration of Glycolytic Activity to Iron-Inactivated Brain by Supply of Energy-Rich Phosphate—To prove convincingly that in the inactivated brain homogenate glyceraldehyde phosphate is limiting solely because of its rôle in the synthesis of adenosine triphosphate (ATP), it was necessary to show that ATP from another source could restore glycolytic activity equally well. Addition of ATP at short intervals has already been shown to have no restoring effect (1). This was not considered conclusive because, as more ATP was added to the brain homogenates, more was destroyed by the active ATPase (adenosine triphosphatase) present.

It was therefore necessary to provide ATP by a continuous reaction from a store of energy-rich phosphate. This was accomplished by the use of phosphocreatine and an enzyme from rabbit muscle which catalyzes the reaction

TABLE IV

Restoration of Glucose Phosphorylation in Iron-Inactivated Brain Homogenates by Addition of Phosphocreatine Enzyme, Phosphocreatine, and Potassium Fluoride

Experimental conditions as in Table I, except that DPN and nicotinic acid amide were added to all experimental tubes, and in the experiments specified 0.1 ml. of 1.2 M potassium fluoride was added.

Additions	Glucose disappearance after 30 min.		
	Normal brain homogenates	Inactivated brain homogenates	Inhibition
	micromoles	*micromoles*	*per cent*
None	7.1	1.6	77
KF + phosphocreatine + phosphocreatine enzyme	8.2	6.4	22
Phosphocreatine enzyme	6.5	1.6	75

$$\text{Phosphocreatine} + \text{adenosine diphosphate} \rightleftharpoons \text{creatine} + \text{adenosine triphosphate}$$

To obtain a valid test, it was necessary to purify this enzyme partially in order to free it of ATPase and triose phosphate dehydrogenase activity. A rabbit muscle extract was dialyzed against distilled water for 5 days and then was precipitated with acetone to remove ATPase activity. The dried acetone powder was extracted with cold water and the clear centrifuged solution fractionated with ammoniacal ammonium sulfate. The fraction obtained at 40 per cent saturation was suitable for the metabolic studies, since it contained no or negligible amounts of ATPase and phosphoglyceraldehyde dehydrogenase. In Table IV the effect of the phosphocreatine enzyme on brain homogenate, inactivated by ferrous sulfate, is recorded. Almost complete restoration of the activity was achieved by addition of the phosphocreatine enzyme, together with phosphocreatine and potassium fluoride which was added to depress ATPase activity. The enzyme without phosphocreatine was without effect, indicating that it was free of triose phosphate dehydrogenase which restores activity in the absence of phosphocreatine.

The high restoring capacity of glyceraldehyde phosphate dehydrogenase from yeast and muscle, the inactivation of this enzyme when added to brain homogenate in the presence of ferrous sulfate, together with the capacity of ATP (when continuously regenerated from phosphocreatine) to restore activity, leave little doubt that an impairment of the coupled phosphorylation of the triose phosphate is the main cause for the inhibitory effect of iron salts on glycolysis of brain homogenate.

Effect of Ferrous Sulfate on Phosphofructokinase—The complete restoration of glycolysis by the addition of purified glyceraldehyde phosphate dehydrogenase pointed to a considerable degree of specificity of the inactivating agent. If present in large excess, other glycolytic enzymes could also have been inactivated under these conditions without affecting the over-all rate of glycolysis, providing their activity was not lowered to the level of the pace-maker.

Earlier experiments on the localization of the inhibition in brain homogenates of mice infected with the Theiler FA virus of mouse encephalomyelitis indicated a slight but consistent inhibition of phosphofructokinase. Addition of purified phosphofructokinase, however, consistently failed to restore glycolytic activity after the inhibitory effect of ferrous sulfate had taken place. Doubts as to the effectiveness of these phosphofructokinase preparations purified from rabbit muscle were removed by testing them on brain homogenates inactivated by incubation at pH 6.3 for 10 minutes at 37°. This procedure has been shown to inactivate phosphofructokinase in mouse brain homogenates (7). As is shown in Table V, the glycolytic activity of an acid-inactivated brain homogenate is fully restored upon addition of phosphofructokinase. The "restoring factor" had no effect under these conditions. The effectiveness of phosphofructokinase in the acid-inactivated brain on the one hand and its lack of restoring capacity in the iron-inactivated brain on the other hand stress the difference in the mechanism of inactivation under these two conditions.

Although it was apparent from these results that the slight inactivation of phosphofructokinase observed in iron-inactivated homogenates is not responsible for the inhibition of the over-all glycolysis, the effect of ferrous sulfate on phosphofructokinase was studied further in the hope of shedding more light on the mechanism of the iron salt effect.

The spectrophotometric determination of phosphofructokinase required the addition of a rabbit muscle fraction which contained restoring factor.

Since, at the time the experiments were carried out, the nature of the restoring factor was unknown, it was decided to measure the phosphofructokinase activity by a chemical method directly in the brain homogenate. In the presence of potassium cyanide, ATP, $MgCl_2$, and an excess of aldolase (which was readily prepared free of restoring factor), the rate of triose phosphate formation was proportional to the amount of phosphofructokinase present. This method followed closely a procedure described by Herbert *et al.* (8) for measuring aldolase activity by determination of alkaline labile phosphate formed. The conditions for the test were as follows: The substrate was fructose-6-phosphate (0.0025 M); to this were added adenosine triphosphate (0.001 M), $MgCl_2$ (0.0035 M), NaF (0.06 M), KCN (0.06 M), and 0.1 ml. of a 2 per cent solution of aldolase recrystallized from rabbit muscle six or seven times (9). The solution of potassium cyanide was made up freshly and was neutralized before use. Brain homogenate suspended in 0.005 M phosphate buffer at pH 7.5 was added in amounts varying from 500 γ to 5 mg. of dry weight.

TABLE V

Inhibition of Glycolysis in Brain Homogenates by Acid Treatment and by Addition of "Inactivating Factor" in Presence of Ferrous Sulfate and Cysteine

Brain homogenate was "acid-treated" by adjusting the pH of the homogenate to 6.3 with 0.01 N HCl, followed by incubation in presence of glucose at 37° for 10 minutes and neutralization with 0.01 N KOH. Phosphofructokinase, "restoring factor," and "inactivating factor" were prepared as described previously (1). The inactivating factor was dialyzed for 3 days against 0.1 M potassium cyanide which was then removed by dialysis against 0.08 M KCl or distilled water for 3 days.

Experiment No.	Tissue preparation	Lactic acid production	Inhibition
		γ	*per cent*
1	Brain homogenate	2300	
2	Acid-treated brain homogenate + restoring factor	100	96
3	As in Experiment 2 + phosphofructokinase	2200	4
4	Brain homogenate + inactivating factor (dialyzed)	1700	26
5	Same as in Experiment 4 + ferrous sulfate (5 γ) and cysteine (600 γ)	560	75

The solutions previously warmed at 38° were mixed, brought to a volume of 2 ml., and finally incubated at 38° for 3 minutes. The reaction was stopped by the addition of trichloroacetic acid. Alkaline labile phosphorus was measured in the filtrate by keeping the solution for 20 minutes at room temperature in 1 N NaOH and correcting for the phosphorus determined on the untreated filtrate. This latter control is necessary for each experiment because of differences in the ATPase activity which leads to the appearance of considerable amounts of inorganic phosphorus from the added ATP, even

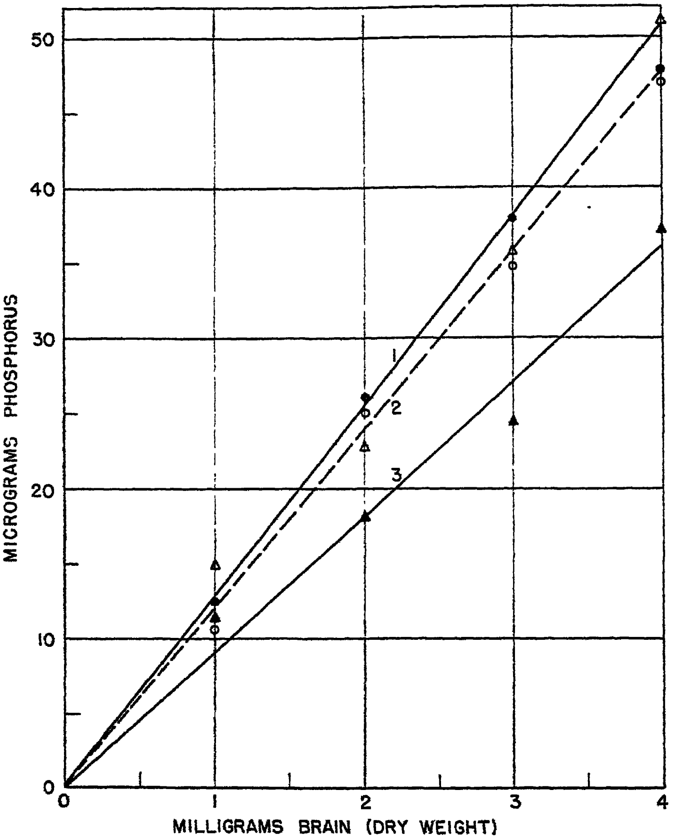

FIG. 1. Inhibition of phosphofructokinase activity in brain homogenates by ferrous sulfate. Experimental conditions as described in the text. Curve 1, phosphofructokinase activity of varying amounts of brain homogenate after incubation for 20 minutes at 38°; Curve 2, ATPase activity of brain homogenate with and without ferrous sulfate; Curve 3, phosphofructokinase activity of brain homogenate incubated for 20 minutes at 38° with 5 γ of ferrous sulfate.

in the presence of sodium fluoride. If corrections are made for the inorganic phosphorus present at the beginning of the experiment, values for the ATPase activity can be obtained from the same data. In Fig. 1 a typical experiment on the relation between brain concentration and phosphofructokinase activity and the effect of ferrous sulfate on the activity of thi enzyme is recorded. Also recorded for comparative purposes is the action of ATPase on ATP in presence of sodium fluoride, calculated from the same data. While the latter enzyme was not affected by ferrous sulfate, phosphofructokinase showed a consistent though slight inhibition. This inhibition could be increased considerably by either prolonging the time of incubation with ferrous sulfate, or by increasing the ferrous sulfate concentration (Fig. 2). It should be noted here that DPN and glucose have a marked protective effect on the triose phosphate dehydrogenase activity but fail to show such an effect on phosphofructokinase.

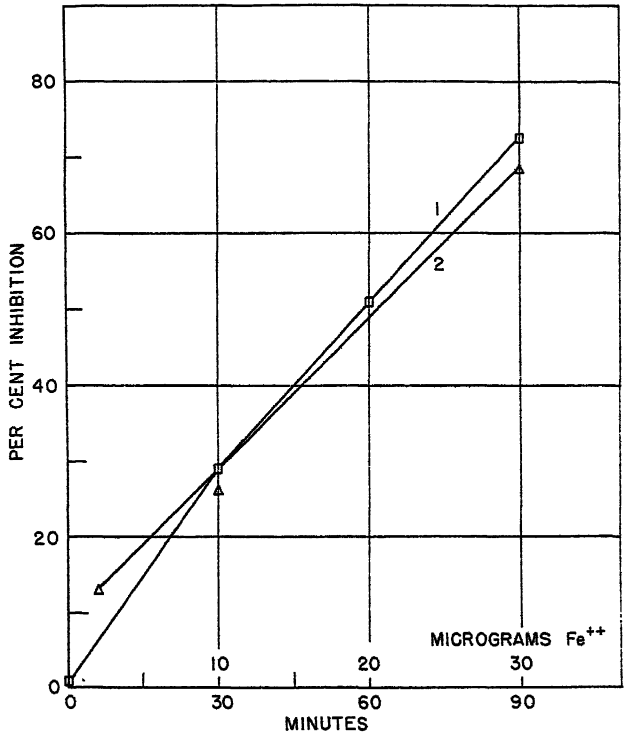

FIG. 2. Effect of time and concentration of ferrous sulfate on inhibition of phosphofructokinase activity in brain homogenates. Curve 1, effect of time of preliminary incubation in the presence of 5 γ of ferrous sulfate on phosphofructokinase activity; Curve 2, effect of varying amounts of ferrous sulfate during preliminary incubation (20 minutes) on phosphofructokinase activity.

Mechanism of Ferrous Sulfate Effect—The dependence of the iron effect on a heat-labile factor, which is present in fresh brain homogenate but which is absent in extracts prepared from acetone-dried brain powder, was previously demonstrated (1). The concentration and purification of this factor were accomplished by centrifugation of the fresh brain homogenate. The tissue particles thus obtained were washed twice with 0.01 M potassium phosphate buffer at pH 7.5. These preparations inhibited glycolysis so strongly that a direct effect could be provoked without a period of preliminary incubation. The preparations were inactivated by dialysis against 0.1 M potassium cyanide at pH 6.0 for 5 days. Full reactivation was accomplished by addition of ferrous sulfate and cysteine (Table V).

TABLE VI

Inhibition of Glycolysis of Brain Homogenates by "Inactivating Factor" and by Proteolytic Enzymes

Crystalline trypsin (8 γ), "inactivating factor," or cathepsin was added before preliminary incubation (20 minutes at 37°); glyceraldehyde phosphate dehydrogenase (GPDase) and 0.1 ml. of 0.035 M fructose-1,6-diphosphate (HDP) were added after preliminary incubation. Time of incubation, 60 minutes.

Experiment No.	Inhibitor	Addition after preliminary incubation	Lactic acid production	Inhibition
			γ	*per cent*
1			2100	
2		GPDase	2000	
3		HDP	2200	
4	Trypsin		1100	48
5	"	GPDase	1800	14
6	"	HDP	1500	28
7	Cathepsin		300	86
8	"	GPDase	1300	38
9	"	HDP	1900	10
10	Inactivating factor		400	81
11	" "	GPDase	1900	10
12	" "	HDP	2300	0

The mechanism by which this iron-activated factor destroys the enzymatic activity of the triose phosphate dehydrogenase and phosphofructokinase and possibly of other enzymes is still obscure. The effect of time and temperature on the activity of this factor and its heat lability suggested an enzymatic reaction. Since glyceraldehyde phosphate dehydrogenase, the enzyme which is inactivated by the brain factor, is of protein nature, a proteolytic destruction was considered as a possible mechanism of inactivation. Attempts made to demonstrate proteolysis have so far been unsuccessful. The methods used (formol titration and determination of acid-soluble tyrosine) may not have been sensitive enough or may be unsuitable for this system. Preliminary experiments with synthetic substrates (benzoylargininamide and glycyldehydroalanine) were negative or inconclusive.[1]

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[1] We wish to thank Dr. M. Levy for the benzoylargininamide, Dr. J. Greenstein for a generous gift of glycyldehydroalanine, and Mr. B. Mandel for assisting in these experiments.

It was found, on the other hand, that 50 γ of crystalline trypsin, added directly to the glycolyzing brain homogenate, or 10 γ of trypsin added during the "preliminary incubation" (1) inhibited glycolysis in a manner similar to that observed with the inactivating factor. The effect of trypsin was also reversed by addition of fructose-1,6-diphosphate or by phosphoglyceraldehyde dehydrogenase.

In view of the possible relation of the inactivating factor prepared from mouse brain to an intracellular proteinase, the effect of a cathepsin on glycolysis was studied. A cathepsin was prepared from beef liver according to the method of Anson (10). The effect of this preparation on glycolysis was identical with that of trypsin or the inactivating factor; namely, the inhibition produced was released by the addition of either fructose-1,6-diphosphate or phosphoglyceraldehyde dehydrogenase (Table VI).

DISCUSSION

Studies on the effect of inhibitors on a complex chain of reactions, such as takes place during the breakdown of glucose to lactic acid, meet with two distinct types of difficulties. On the one hand, the rate of the over-all reaction is governed by the slowest of the enzymatic reactions and so the effect of inhibitors on the enzymes present in excess may not be readily noticed. On the other hand, an enzyme which is present in excess, as far as the utilization of an intermediary is concerned, may be limiting with regard to another function such as the production of energy-rich phosphate.

In this study on the effect of iron salts on glycolytic activity, the apparent paradox was observed that inhibition of the phosphoglyceraldehyde dehydrogenase manifested itself most strikingly in the phosphorylation of glucose to fructose-1,6-diphosphate, which reaction occurs at a much earlier stage of glycolysis. When fructose-1,6-diphosphate was added, the inhibition of lactic acid production was counteracted. This complete masking of the actual localization of the inhibition can be explained as follows: For the phosphorylation of glucose, energy-rich phosphate has to be supplied. The partial inhibition of the glyceraldehyde phosphate dehydrogenase with its coupled phosphorylation leads to a decreased formation of ATP. This, in turn, results in an inhibition of glucose phosphorylation and, therefore, less substrate (glyceraldehyde phosphate) for the glyceraldehyde phosphate dehydrogenase is formed. Thus, the production of ATP is further reduced. This vicious circle leads to an amplification of the inhibitory effect on over-all glycolysis which is out of proportion to the effect on the single enzyme.

The production of lactic acid from fructose-1,6-diphosphate is hardly affected, most likely because an enzymatic step, other than the oxidation of glyceraldehyde phosphate to phosphoglyceric acid, is the limiting factor of the over-all reaction.

The above mechanism can be shown in simplified form as follows:

(*a*) Glucose + ATP → fructose-1,6-diphosphate

(*b*) Glyceraldehyde phosphate ⇌ phosphoglyceric acid + ATP

(*c*) Phosphoglyceric acid ⇌ lactic acid

It is clear that, if reaction (*c*) is limiting, a partial inhibition of reaction (*b*) will not be observed if reaction (*b*) plus (*c*) is measured; if reaction (*a*) plus (*b*) plus (*c*) is measured and reaction (*a*) becomes limiting because of its dependence on reaction (*b*), a pronounced inhibition may result.

It is felt that such considerations have a bearing on a number of observations on the effect of inhibitors reported in the literature. For instance, it has been stressed by Shorr (11) that concentrations of iodoacetic acid which suffice to inhibit anaerobic glycolysis are ineffective aerobically. Since glyceraldehyde phosphate dehydrogenase among the glycolytic enzymes is most highly susceptible to this inhibitor, it might be assumed that its partial inhibition may be observed only under anaerobic conditions, while aerobically ATP can be produced from oxidation of pyruvic acid, masking the inhibition. This explanation would eliminate the necessity to postulate other pathways of glucose breakdown.

The possible relation of the inactivating factor to a proteolytic enzyme has been considered previously (1). A serious objection to this assumption lay in the apparent specificity of the reaction for one of the glycolytic enzymes only. It has now been demonstrated that the inactivating factor of brain homogenate affects at least one additional glycolytic enzyme, although to a lesser extent. Since both trypsin and cathepsin inhibit glycolysis in the same manner as the inactivating factor, it must be assumed that the glycolytic enzymes vary considerably in their susceptibility to proteolytic enzymes. These observations with known proteolytic enzymes shift the problem of specificity from the inactivating factor to the substrate. The importance of substrate specificity has been stressed by Bergmann and Fruton (12) in their studies on hydrolysis of synthetic peptides. A better knowledge of the susceptibility of different glycolytic enzymes to the series of known proteolytic enzymes, as well as of their protection from proteolysis by substrates and coenzymes, may throw some light on the construction of the enzyme protein molecule and its active centers.

SUMMARY

1. The inhibiting effect of ferrous sulfate on brain glycolysis has been localized to the coupled phosphorylation catalyzed by the phosphoglyceraldehyde-oxidizing enzyme. Partial inhibition of this enzyme leads to in-

hibition of the synthesis of ATP which is necessary for the formation of fructose-1,6-diphosphate from glucose.

2. The phosphoglyceraldehyde-oxidizing enzyme prepared from yeast or muscle restores the glycolytic activity to a brain homogenate which has been inactivated by ferrous sulfate or by the addition of proteolytic enzymes.

3. The inactivating factor loses its activity after dialysis against 0.1 M KCN for 5 days. Full reactivation can be accomplished by the addition of ferrous sulfate and cysteine.

4. The interpretation of the effect of inhibitors on complex reactions involving several enzymes is discussed.

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THREONINE-SERINE ANTAGONISM IN SOME LACTIC ACID BACTERIA

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WITH THE TECHNICAL ASSISTANCE OF MELBA BREEDLOVE SAMPLE

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Various investigators have reported antagonism between amino acids in the nutritional requirements of microorganisms. Snell and Guirard (1) have reported the inhibitory effect of glycine, serine, β-alanine, and threonine on the utilization of alanine. Gladstone (2), using *Bacillus anthracis*, found that certain amino acids prevented growth when added singly to a medium capable of supporting growth without them. He found valine to be inhibitory to leucine utilization and vice versa. Similar interrelationships were found for valine and threonine, and threonine and serine.

In preliminary investigations by the authors it was noted that serine assays with *Lactobacillus delbrueckii*, *Lactobacillus casei*, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* P-60 varied greatly. Standard serine curves by these four organisms were characterized by having a lag section in the curve at low concentrations of serine, and variation in results seemed to follow the extent of the initial lag section of the curve; *i.e.*, the assay values obtained with the organism having the greatest lag in the standard serine curve varied over a wider range than assay values obtained with those organisms exhibiting the smallest lag in the standard curve.

From the work of Gladstone (2) with *Bacillus anthracis* and from the sigmoidal shape of the standard serine curves, it was thought possible that the lag in the standard curve was due to the inhibition of serine utilization by threonine. This paper presents the results of experiments in a study of the inhibitory effect of threonine on serine utilization and also the inhibitory effect of serine on threonine utilization by some lactic acid bacteria.

Procedure

Stock cultures of *Lactobacillus delbrueckii* 9595, *L. casei*, *Streptococcus faecalis* R (formerly known as *Streptococcus lactis* R), and *Leuconostoc mesenteroides* P-60 were carried on a solid medium containing 1.5 per cent agar, 1 per cent glucose, and 1 per cent yeast extract. *L. arabinosus* 17-5 was carried on a solid medium containing 0.8 per cent agar, 1 per cent peptonized milk. 1 per cent tryptone, and 200 ml. of filtered tomato juice per liter of medium (3). The inoculum medium of Stokes and Gunness (4) was used for all the organisms except *L. arabinosus*. The tomato juice medium given above with the agar omitted was used for inoculum media for this organism.

The metabolites to be studied (adjusted to pH of the medium) were added to tubes and the volume was adjusted to 5 ml. with distilled water. 5 ml. of appropriate double strength media were then added. The tubes were plugged with non-absorbent cotton and autoclaved at 15 pounds pressure for 10 minutes. After being cooled, each tube was inoculated with 1 drop of suspension of organisms which was prepared by washing a 10 ml. 18 to 24 hour culture twice with physiological saline, and then diluted to a final volume of 100 ml. The tubes were placed in a water bath at 35° and incubated for 96 hours for *Leuconostoc mesenteroides* and 72 hours for the other organisms. At the end of the incubation period, the lactic acid produced

TABLE I

Composition of Leuconostoc mesenteroides, Media A and B*

Constituent	Final concentration per 10 ml. tube		Constituent	Final concentration per 10 ml. tube	
	Medium A	Medium B		Medium A	Medium B
	mg.	*mg.*		*mg.*	*mg.*
L-Arginine monohydrochloride	2	1	Adenine	0.12	0.10
DL-Alanine	10	2	Guanine	0.12	0.10
DL-Aspartic acid	20	4	Uracil	0.12	0.10
L-Cystine	2	1	KH_2PO_4	10	10
Glycine	1	1	K_2HPO_4	10	10
DL-Glutamic acid	8	4	$MgSO_4 \cdot 7H_2O$	2	2
L-Histidine monohydrochloride	1	1	NaCl	0.10	0.10
DL-Isoleucine	2	2	$MnSO_4 \cdot 4H_2O$	0.10	0.10
DL-Leucine	2	2	$FeSO_4 \cdot 7H_2O$	0.10	0.10
L-Lysine monohydrochloride	2	1		γ	γ
DL-Methionine	1	2	Biotin	0.05	0.01
DL-Norleucine		2	Calcium pantothenate	20	2
L-Proline	1	1	Choline chloride		25
DL-Phenylalanine	1	2	Folvite†	0.04	0.04
L-Tryptophan	0.5	1	Inositol		25
L-Tyrosine	1	1	Nicotinic acid	20	2
DL-Valine	2	2	*p*-Aminobenzoic acid	0.01	0.01
Glucose	200	200	Pyridoxine hydrochloride	16	16
Sodium acetate (anhydrous)	120	60	Riboflavin	20	2
NH_4Cl	60	30	Thiamine hydrochloride	10	5

* Lyman and Kuiken, unpublished.

† Lederle Laboratories Division, American Cyanamid Company.

was titrated with 0.1 N NaOH to pH 7.0, as determined by an indicating pH meter. The DL form of both serine and threonine was used in all the work presented. All concentrations of serine and threonine given in this paper refer to the DL form unless otherwise designated.

The medium of Stokes, Gunness, Dwyer, and Caswell (5) was used for *Lactobacillus delbrueckii* with the exception that pyridoxine hydrochloride (20 γ per 10 ml. tube) was used instead of pyridoxamine. For *Streptococcus faecalis*, Medium II of Baumgarten, Mather, and Stone (6) was employed with the xanthine omitted. The *L. casei* medium as described by McMahan and Snell (7) with xanthine omitted was used for this organism. The media for *Leuconostoc mesenteroides* are given in Table I. Medium A of Table I is from unpublished work of Kuiken and Lyman and was used in obtaining data for *Leuconostoc mesenteroides* in Figs. 1 and 5. Medium B was employed for the other experiments with this organism. The medium of Lyman *et al.* (3) was used for *L. arabinosus* with the omission of the tomato eluate and the addition of folic acid.

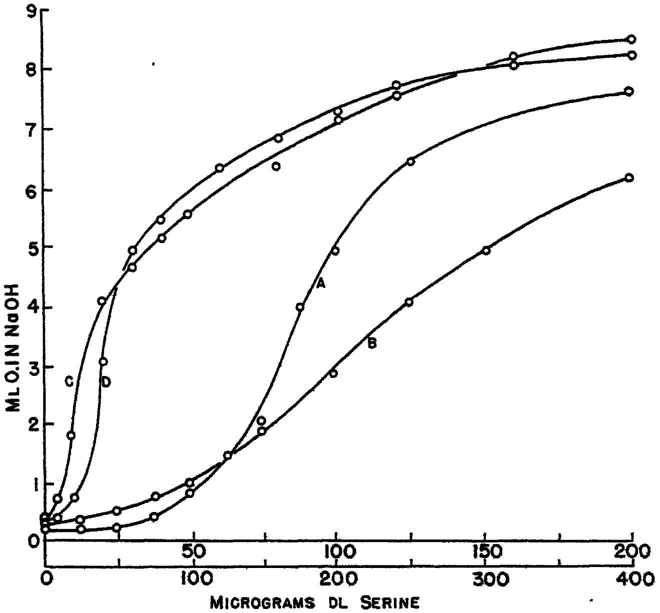

FIG. 1. Standard serine curves. Curve A, *Lactobacillus delbrueckii*, 0 to 400 γ of serine; Curve B, *Lactobacillus casei*, 0 to 400 γ of serine; Curve C, *Streptococcus faecalis*, 0 to 200 γ of serine; and Curve D, *Leuconostoc mesenteroides*, 0 to 200 γ of serine.

Results

Standard serine curves with their characteristic lag at low levels of serine are shown in Fig. 1 for four serine-requiring lactic acid bacteria. Such curves are obtained in the presence of 2 mg. of threonine per 10 ml. tube, which is the amount of this metabolite present in basal media used for serine assays (4, 6). The lag section of Curves A and B, extending to 100 γ, is approximately ten times greater than the lag section of Curves C and D.

The inhibitory effect of threonine on the utilization of serine and the resulting lag in the standard serine curves are clearly demonstrated in Figs. 2 to 5. *Lactobacillus delbrueckii* (Fig. 2) and *L. casei* (Fig. 3) do not require threonine for growth and the omission of this metabolite from the media for these two organisms completely removes the lag in the growth curves.

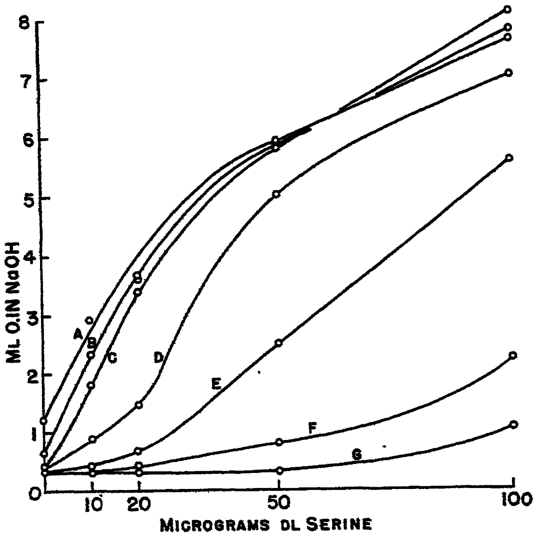

FIG. 2. Effect of threonine on serine utilization by *Lactobacillus delbrueckii*. Curve A, no threonine; Curve B, 100 γ of DL-threonine per tube; Curve C, 200 γ of DL-threonine per tube; Curve D, 500 γ of DL-threonine per tube; Curve E, 1000 γ of DL-threonine per tube; Curve F, 2000 γ of DL-threonine per tube; Curve G, 4000 γ of DL-threonine per tube.

With standard serine Curves A, B, and C of Fig. 2, obtained in the presence of 0, 100, and 200 γ of threonine, it is to be noted that Curve A falls below Curves B and C for higher growth. This would suggest that threonine is stimulatory for *L. delbrueckii* at higher growth levels. However, in other experiments this was not apparent and was not noticed with *L. casei* (Fig. 3).

Streptococcus faecalis and *Leuconostoc mesenteroides* both require threonine and it is therefore impossible to remove the lag sections completely by the omission of threonine. From Figs. 4 and 5 for these two organisms, it is apparent that the lag is reduced by diminishing the threonine, but it seems that for *L. mesenteroides* and possibly *Streptococcus faecalis* some other factors are responsible for some of the lag. These results with *L. mesenteroides* are similar to those of Horn, Jones, and Blum (8) in which they found it impossible to remove the lag completely from the standard threonine curves by lowering the serine content of the basal medium.

Inhibition Ratios of Threonine to Serine—A quantitative study of the inhibition of serine utilization is given in Table II. For this study the "antibacterial index" method of McIlwain (9) was used. This particular approach has been extensively used by Shive and coworkers (10–12) in studying competitive analogue-metabolite growth inhibitions. For data in Table II a wide range in concentration of threonine was used and serine was added to give the ratios as indicated. The ratio of threonine to serine at which the titrations are equal to the blank titrations (no serine added) is taken as the "antibacterial index" or inhibition ratio. For *Lactobacillus delbrueckii* the inhibition ratio is between 100 and 200. At the ratio of 100 there was growth at higher levels of threonine and serine. This indicated that at this ratio there was still serine present for growth above the ratio of inhibition. At low levels this amount of serine was so small that no response could be detected. However, at a ratio of 200 the inhibition was complete for the concentrations of threonine and serine used. An experiment repeated with the 100 and 200 γ serine levels, with varying amounts of threonine, gave in each case a ratio of 150. *Lactobacillus casei* was completely inhibited at a ratio of between 50 and 100, as shown by Table II. A repetition of the experiment with 100 and 200 γ of serine and varying concentrations of

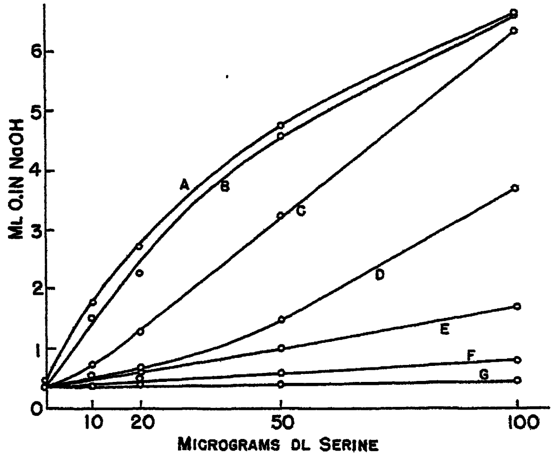

FIG. 3. Effect of threonine on serine utilization by *Lactobacillus casei*. Curve A, no threonine; Curve B, 100 γ of DL-threonine per tube; Curve C, 200 γ of DL-threonine per tube; Curve D, 500 γ of DL-threonine per tube; Curve E, 1000 γ of DL-threonine per tube; Curve F, 2000 γ of DL-threonine per tube; Curve G, 4000 γ of DL-threonine per tube.

threonine gave an inhibition ratio of 75. Inhibition ratios greater than 1000 for *Leuconostoc mesenteroides* and 2000 for *Streptococcus faecalis* are in-

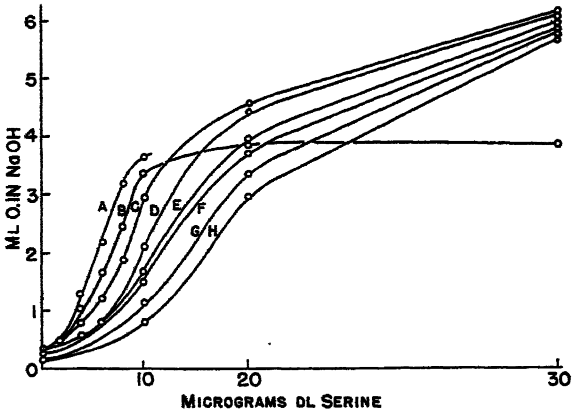

FIG. 4. Effect of threonine on serine utilization by *Streptococcus faecalis*. Curve A, 25 γ of DL-threonine per tube; Curve B, 50 γ of DL-threonine per tube; Curve C, 100 γ of DL-threonine per tube; Curve D, 200 γ of DL-threonine per tube; Curve E, 500 γ of DL-threonine per tube; Curve F, 1000 γ of DL-threonine per tube; Curve G, 2000 γ of DL-threonine per tube; Curve H, 4000 γ of DL-threonine per tube.

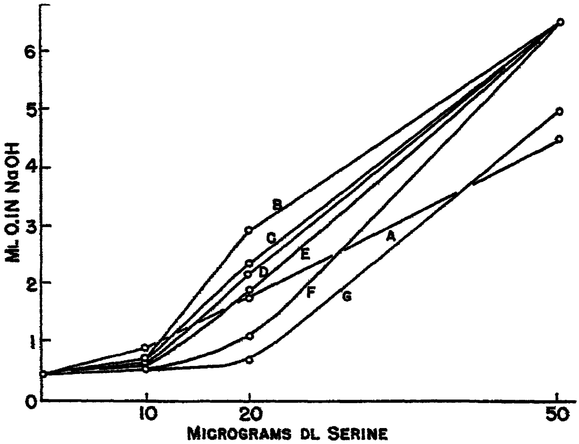

FIG. 5. Effect of threonine on serine utilization by *Leuconostoc mesenteroides*. Curve A, 50 γ of DL-threonine per tube; Curve B, 100 γ of DL-threonine per tube; Curve C, 200 γ of DL-threonine per tube; Curve D, 500 γ of DL-threonine per tube; Curve E, 1000 γ of DL-threonine per tube; Curve F, 2000 γ of DL-threonine per tube; Curve G, 4000 γ of DL-threonine per tube.

dicated by Table II. By extrapolation of this data, ratios of 1100 to 1400 and 2600 to 2800 are obtained. Analogous experiments with 10 and 20 γ

TABLE II

Inhibition of Serine Utilization by Threonine

Organism	Threonine	Titration, ml. of 0.05 N NaOH				
		Ratio of threonine to serine				
		No serine	200	100	50	25
	mg.					
Lactobacillus delbrueckii	0.20	0.70	0.60	0.70	1.05	1.00
	0.50	0.60	0.60	0.60	0.80	0.80
	1.00	0.50	0.55	0.50	0.80	1.36
	2.00	0.52	0.52	0.50	0.74	0.92
	4.00	0.50	0.54	0.55	0.84	1.05
	10.00	0.55	0.60	0.58	1.05	1.50
	20.00	0.56	0.60	0.65	1.20	2.55
	40.00	0.70	0.70	1.20	2.28	13.75
		Ratio of threonine to serine				
		No serine	100	50	25	10
Lactobacillus casei	0.20	0.85	0.71	0.83	0.91	1.00
	0.50	0.70	0.70	0.82	0.85	1.45
	1.00	0.70	0.70	0.80	0.81	2.30
	2.00	0.70	0.70	0.61	0.80	1.68
	4.00	0.68	0.66	0.71	0.67	2.37
	10.00	0.68	0.68	0.80	0.84	3.60
	20.00	0.70	0.72	0.90	1.13	9.08
		Ratio of threonine to serine				
		No serine	1000	400	200	100
Leuconostoc mesenteroides P-60	0.50	0.14	0.80	0.94	0.90	0.94
	1.00	0.72	1.00	1.00	0.97	3.78
	2.00	0.80	0.89	0.95	1.33	4.17
	4.00	0.78	0.81	1.15	2.00	8.72
	10.00	0.79	0.90	2.00	7.80	10.80
	20.00	0.85	1.10	3.96	13.10	14.80
		Ratio of threonine to serine				
		No serine	2000	1500	1000	800
Streptococcus faecalis R	2.00	0.04	0.14	0.34	0.48	0.59
	4.00	0.04	0.34	0.54	0.34	0.62
	10.00	0.00	0.56	0.84	1.32	2.09
	20.00	0.00	0.84	1.51	3.49	4.14
	40.00	0.00	1.84	3.34	7.38	8.84

of serine gave inhibition ratios of 1500 for *Leuconostoc mesenteroides* and 2000 to 4000 for *Streptococcus faecalis*.

Inhibition Ratios of Serine to Threonine—If threonine, because of its similarity in structure to serine, can block the utilization of serine, it should also be feasible that serine could block the utilization of threonine. Data in Table III clearly demonstrate that serine can effectively inhibit the utilization of threonine by some threonine-requiring lactic acid bacteria that have been used for the assay of threonine. An inhibition ratio of serine to threonine of approximately 600 is obtained for *Streptococcus faecalis* by interpolation and extrapolation of data given in Table III. By the same method, the best values indicate an inhibition ratio of 200 for *Leuconostoc mesenteroides* and between 400 and 600 for *Lactobacillus arabinosus*.

TABLE III

Inhibition of Threonine Utilization by Serine

Organism	Threonine per tube	Serine per tube, mg.										
		0	0.4	1.0	2.0	4.0	10.0	20.0	40.0	60.0	80.0	100.0
		Titration, ml. of 0.1 N NaOH										
	γ											
Streptococcus faecalis	20	0.95	2.86	2.85	2.82	2.74	1.34	0.0				
	100	0.62	7.60	7.42	7.30	7.30	6.30	5.50	4.12	0.78		
	200	0.56	8.62	8.70	8.50	8.52	8.30	8.25	7.70	6.45	4.20	2.00
Leuconostoc mesenteroides P-60	20	0.30	0.91	0.30	0.30	0.26	0.32					
	100	0.32	4.18	4.02		3.85	0.84	0.33	0.32			
	200	0.68	5.80	5.20	4.80	3.68	3.30	3.18	0.30	0.30	0.35	
Lactobacillus arabinosus	100	10.40			6.35	4.82	2.81	2.04	1.81	1.70	1.72	1.72

DISCUSSION

The antagonistic effect of threonine on the utilization of serine has some implications as to the reliability of the microbiological determination of serine. The technique of heavily dosing the medium with all constituents, other than the one to be assayed, is usually sufficient to eliminate the effect of metabolites added as samples. However, with *Lactobacillus delbrueckii* and *Lactobacillus casei*, the addition of excess threonine (2 mg. per 10 ml. tube) is not desirable. The large lag section in the standard serine curve by these two organisms obtained in the presence of high concentration of threonine is objectionable because in the lag section the growth response to added serine is relatively small. This decreases the sensitivity and limits the accuracy of assay values taken in the lag section.

Also in serine assays with *Lactobacillus delbrueckii* and *Lactobacillus casei*, the effect of threonine added as a sample is important even in the presence of 2 mg. of threonine per tube. Curve F (2 mg. of threonine per tube) and Curve G (4 mg. of threonine per tube) of Fig. 2 demonstrate adequately that the addition of threonine, in excess of the 2 mg. usually used in assay procedure, causes a decrease in titration at a given level of serine. For example, with Curves F and G there is a difference in titration of 0.5 ml. at 50 γ of serine and 1.2 ml. at 100 γ of serine. Experimental data not shown, but which represent an extension of these same two curves, Curves F and G, gave differences in titration of 4.25 ml. at 200 γ of serine and 3.75 ml. at 300 γ of serine. It is true that these differences were obtained by adding 2 mg. of threonine in excess of the amount used in Curve F, but Table IV shows that such conditions are possible in the serine assay by *Lactobacillus delbrueckii*.

TABLE IV

Comparison of Amount of Threonine Added by Several Samples in Assay for Serine by Lactobacillus delbrueckii

Sample	L-Serine	L-Threonine	Assay range, DL-serine	Sample for assay range	L-Threonine added as sample for assay range
	per cent	*per cent*	γ	*mg.*	γ
Dried cheese whey	0.04 (6)	1.9 (6)	0–400	0–50	0–950
Casein	6.8 (6)	4.5 (6)	0–400	0–3	0–135
Silk fibroin	11.4 (13) 14.5 (4)	1.3 (13)	0–400	0–2	0–26

The figures in parentheses refer to the bibliography.

Table IV gives three samples that vary in the ratio of the threonine to the serine content. It can be noted from Table IV that with such a sample as dried cheese whey, in which the threonine content is approximately five times greater than the serine content, a maximum of 950 γ of L-threonine would be added in an assay range of 0 to 400 γ of serine (Curve A, Fig. 1). This quantity of threonine added as a sample approaches very closely the difference in L-threonine content of the media used in obtaining Curves F and G, Fig. 2. Such additions of threonine would have a pronounced effect on the response of the organism to serine and as a result would give low assay values which would become increasingly lower as the amount of the sample was increased. Silk fibroin on the other hand has a much higher serine content than threonine and therefore the amount of threonine added would be very small, as is shown by Table IV. Thus with this sample the effect of added threonine as a sample would be at a minimum.

Casein is more representative of most of the samples on which serine and threonine values have been reported (6, 13). Although most of these samples contain a little more serine than threonine, it is evident from Table IV and Fig. 2 that the additions of threonine as a sample would have the same effect on the assay as with dried cheese whey, only to a lesser extent.

The data presented indicate that a good assay procedure for serine, in samples containing no threonine, would be the use of *Lactobacillus delbrueckii* with a basal medium free of threonine.

The use of *Streptococcus faecalis* or possibly *Leuconostoc mesenteroides* shows more promise of being adapted to a serine assay than any of the other organisms mentioned in this paper, when the effect of factors other than threonine is more clearly understood. With *Streptococcus faecalis* the lag section is relatively short and the upper limit of the assay range is low. This allows the use of small quantities of sample which minimizes the effect of threonine added as a sample. These possibilities are being investigated in this laboratory and will be reported later.

SUMMARY

Data have been presented to show the antagonistic effect of threonine on the utilization of serine. Increasing amounts of threonine added to basal media have been shown to cause a decrease in growth at a given level of serine. Ratios of threonine to serine of approximately 150, 75, 1100 to 1400, and 2000 to 4000 completely prevented growth of *Lactobacillus delbrueckii, Lactobacillus casei, Leuconostoc mesenteroides,* and *Streptococcus faecalis* under the conditions presented. Implications of the antagonistic effect of threonine on serine utilization as related to the microbiological assay methods for serine have been discussed.

A few data have also been presented concerning the antagonistic effect of serine on the utilization of threonine. Ratios of serine to threonine of 600, 200, and 400 to 600 have been shown to inhibit effectively the growth of *Streptococcus faecalis, Leuconostoc mesenteroides,* and *Lactobacillus arabinosus.*

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THE EFFECT OF SURFACE-ACTIVE SUBSTANCES ON THE FUCHSIN REACTION OF HIGHER FATTY ALDEHYDES*

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Higher fatty aldehydes are present in considerable quantity in the lipide fraction of muscle and brain and may be intermediates in lipide metabolism (1, 2). The meager information concerning the quantitative distribution of the higher fatty aldehydes in tissues has been acquired exclusively by quantitative evaluation of the Schiff reaction, as in the procedure of Feulgen and Grünberg (3) or modifications of it (4). Serious doubt as to the reliability of the method arose when it was found that added palmitaldehyde or stearaldehyde or their acetals could not be estimated quantitatively in tissue extracts (4).

The experiments reported in this paper show that the result of the quantitative fuschsin reaction for the determination of higher fatty aldehydes depends to a large degree on the presence of surface-active lipides in the tissue extract. Naturally occurring lipides or synthetic surface-active agents inhibit the color development if added at the beginning of the reaction, and destroy the color already formed if added later. This effect of surface-active agents can be suppressed to a large degree by reducing the water content of the medium through the use of a high concentration of acetic acid.

EXPERIMENTAL

The Schiff reaction was employed in three forms, two at low and one at high concentrations of acetic acid. In all expriments palmitaldehyde glyceryl acetal was used as reference substance (4).

Reactions at Low Acetic Acid Concentrations (Reactions S. F. and S. F. HCl)—The Schiff reaction for the determination of higher fatty aldehydes as carried out previously in our laboratory (4) differed in three respects from the procedure used by Feulgen and Grünberg (3): (*a*) 1 ml. of 1 N HCl was added to the reaction mixture consisting of 10 ml. of fuchsin reagent, mercuric chloride solution, and 1 ml. of glacial acetic acid containing the compounds or tissue components to be tested. It was found that increased

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* The higher fatty aldehydes, IV. This work was supported by grants from the Josiah Macy, Jr., Foundation and the United States Public Health Service.

acidity resulted in a greater precision. (*b*) The color was developed at 37° for 18 to 24 hours. (*c*) The color complex was extracted with capryl (4, 5) instead of amyl alcohol (3). In the present experiments this procedure (Reaction S. F. HCl) was compared with a procedure (Reaction S. F.) in which no HCl was added. The reaction mixtures contained 8.9 per cent (Reaction S. F.) and 8.1 per cent (Reaction S. F. HCl) acetic acid respectively.

Reaction at High Acetic Acid Concentration (*Reaction S. A. A.*)—2 gm. of basic fuchsin (National Aniline Division) were dissolved in 50 ml. of glacial acetic acid. 10 gm. of sodium bisulfite, 100 ml. of 0.1 N HCl, and 50 ml. of water were added in succession. The reagent was used after it had stood for several hours. The bisulfite did not decolorize the solution appreciably; the final reagent retained a reddish brown color. To 1 ml. of glacial acetic acid containing the compounds or tissue components to be tested 2 ml. of glacial acetic acid and 1 ml. of fuchsin reagent were added. The color was developed in sealed glass tubes (9 mm. inside diameter, 10 ml. capacity) at 50° for 18 to 20 hours. When cool, the sealed tubes were opened, 2 ml. were transferred to a 25 ml. graduated cylinder (glass-stoppered), and 10 ml. of an aqueous solution were added, containing 5 gm. of sodium bisulfite and 5 ml. of concentrated HCl in 100 ml. In the blank samples the color faded within 10 minutes to a light yellow. The solutions were extracted with 10 ml. of capryl alcohol exactly 10 minutes after the addition of the sulfite solution, and the alcoholic solution was cleared by centrifuging as described previously (4).

Substrates—The preparation of palmitaldehyde and stearaldehyde and their acetals was described previously (4). As synthetic surface-active substances the non-ionic detergents, Tweens and Spans, of the Atlas Powder Company (mono- and polyesters of sorbitan with long chain fatty acids and their polyalkylene derivatives) were used. These substances produced small and consistent color values. The crude egg yolk phosphatides were prepared according to the method of Feulgen and Grünberg (3) and dried to constant weight. The samples gave fuchsin color values (Reaction S. F. HCl) corresponding to as much as 720 mg. of palmitaldehyde per 100 gm. of lipide. In the experiments with brain extract the residue of an alcohol-ether extract of finely minced brain was dissolved in the appropriate amount of glacial acetic acid.

In all experiments reported in this paper the color density was determined with a Coleman junior spectrophotometer, model 6, in cuvettes No. 6-302 at 545 mμ. The values obtained in Reaction S. A. A. were doubled, since only half of the reaction mixture was extracted with capryl alcohol.

RESULTS AND DISCUSSION

The addition of Span 20, egg yolk phosphatides, or brain lipides to palmitaldehyde or its glyceryl acetal resulted in an inhibition of the color development if the Schiff reaction was carried out in a medium of approximately 90 per cent water (Reactions S. F. and S. F. HCl) (Table I). By carrying out the Schiff reaction in a medium containing 80 per cent acetic acid (Reaction S. A. A.) the effect of the surface-active agents was minimized and the recoveries of added aldehyde or acetal amounted to 66 to 100 per cent. The recovery of total color resulting from the aldehyde or acetal plus that from various compounds which were added amounted in Reactions S. F. and S. F. HCl to 0 to 74 per cent and in Reaction S. A. A. to 88 to 100 per cent.

TABLE I

Schiff Reaction of Palmitaldehyde and Its Glyceryl Acetal in Presence of Lipides and Synthetic Surface-Active Agents

Source of aldehyde	Amount	Addition	Amount	Schiff reaction* optical density			Analytical recovery		
				Reaction S. F.	Reaction S. F. HCl	Reaction S. A. A.†	Reaction S. F.	Reaction S. F. HCl	Reaction S. A. A.
	γ		mg.				per cent	per cent	per cent
Palmitalde-	30				0.11	0.24			
hyde	30	Span 20	4		0.02	0.23		18	96
	30	" 20	8		0	0.22		0	93
	40			0.16	0.16	0.24			
	40	Span 20‡	20	0.09	0.03	0.25	56	20	105
	35					0.26			
	35	Phosphatides	16			0.20			77
	20			0.12	0.09				
	20	Phosphatides	20	0.05	0.02		40	22	
	20				0.07	0.12			
		Brain lipides	14§		0.07	0.24			
	20	" "	14		0.10	0.32		44	66
Palmitalde-	82			0.31	0.28	0.68			
hyde gly-	82	Span 20	20	0.02	0.05	0.64	6	18	94
ceryl	30					0.26			
acetal	30	Phosphatides	15			0.26			100
	40				0.16				
	40	Phosphatides	1		0			0	
	61					0.54			
		Brain lipides	11			0.32			
	61	" "	11			0.78			85

* In all experiments in which Span 20 or egg phosphatides were added the values are corrected for the densities given by these substances alone.

† Optical density for palmitaldehyde glyceryl acetal: 20.4 γ, 0.17; 40.8 γ, 0.35; 61.2 γ, 0.52; 81.6 γ, 0.66; 102 γ, 0.82; for palmitaldehyde 20.2 γ, 0.15; 40.5 γ, 0.30; 81 γ, 0.54; 101 γ, 0.66.

‡ Span added 5 hours after the start of the reaction.

§ Weight of wet brain.

The color reaction at high acetic acid concentration is approximately twice as sensitive as that carried out at low acetic acid concentrations. Of the latter reactions the one with the higher acidity (Reaction S. F. HCl) is slightly less sensitive and more susceptible to the action of surface-active agents than the one with lower acidity (Reaction S. F.).

In agreement with previous observations, equivalent quantities of different aldehydes or of the same aldehyde on different days did not yield the same color density. The same aldehyde sample tested in different concentrations does not follow Beer's law (*cf.* foot-note, Table I) probably because of the difficulty in obtaining monomeric aldehydes, and therefore the results of experiments carried out on these substrates are variable. With acetal a better linear relation is obtained (*cf.* foot-note, Table I).

In an experiment reported in Table I, 1 mg. of egg yolk phosphatide inhibited completely the color developed by 40 γ of acetal (Reaction S. F. HCl). However, when 0.5 mg. of phosphatide was added, definite inhibition of color development was found in some experiments, but in others the color values coincided (within the error of the method) with the control value of acetal alone. This finding may explain the result reported previously (4) that addition of 1 mg. of egg yolk phosphatides, prepared according to Feulgen and Grünberg, does not affect the 18 hour color value of acetal. In that experiment the phosphatide preparation used was not dried to constant weight and the actual amount may have been considerably less than 1 mg.

The effect of surface-active agents can also be demonstrated on the aldehydes present in tissue extracts. 20 mg. of Tween 85 were added to a solution of brain lipides in glacial acetic acid. Measurements at different time intervals from 30 minutes up to 18 hours after mixing the reagents gave color values corresponding to 10 to 16 per cent of the simultaneously determined control values. Similar results were obtained in experiments in which Span 20 was used as the surface-active agent.

The addition of detergent after full color had developed with palmitaldehyde (Reaction S. F. HCl) caused fading. On the other hand if Reaction S. A. A. was used, full color was obtained when the detergent was added 5 hours after mixing of the reagents (Table I).

The effect of surface-active agents on the Schiff reaction of the higher fatty aldehydes in aqueous medium supports the point of view expressed previously (4), that analytical results obtained with the fuchsin method probably do not represent the true concentrations of higher fatty aldehydes in tissue extracts. The fact that consistent values have been obtained may be a reflection of relatively constant ratios between higher fatty aldehydes and surface-active lipides. An apparent variation, found by the fuchsin method in aqueous media, in the aldehyde concentrations in a tissue under physiological or pathological conditions may be the result of a change in concentration of aldehydes, of surface-active lipides, or of both (5, 6). The form in which the aldehydes are present in the extracts is not known with certainty, and the lipide composition occurring in an extract obtained from tissues with an organic solvent cannot be duplicated experimentally. It is, therefore, difficult to decide how well model experiments with detergents, free aldehydes, and acetals approximate conditions in tissue extracts. But it appears that the new procedure elaborated for the use of the Schiff reaction as presented in this paper may eliminate one of the potential errors in the determination of the higher fatty aldehydes in tissue extracts

SUMMARY

Naturally occurring lipides and synthetic surface-active substances inhibit the color development of the higher fatty aldehydes and their acetals in the fuchsin test as proposed by Feulgen and used in the original or modified form by others. If the surface-active substances are added after color has developed, rapid fading occurs. Addition of synthetic detergents in the determination of aldehydes present in tissue lipides also suppresses the development of color to a marked degree. These findings cast serious doubt on the usefulness of the fuchsin method for the quantitative determination of the higher fatty aldehydes as carried out with the Feulgen method or its modifications.

The effect of surface-active, naturally occurring or synthetic agents is suppressed to a large degree if the Schiff reaction is carried out in a medium containing a high concentration of acetic acid.

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THE PEPTIDASES OF SKELETAL, HEART, AND UTERINE MUSCLE*

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Early investigators of peptidases ascribed the hydrolysis of simple peptides to a relatively small number of enzymes. In recent years, it has become evident that there must be many different peptidases whose specificity is dependent on the nature of the constituent amino acids as well as on the presence of free amino or carboxyl groups. Most of the early studies were confined to a few animal tissues with intestinal mucosa as the main source of these enzymes. It is obviously desirable to examine other tissues for the presence of proteolytic enzymes.

In this study, it will be shown that peptidases of high activity are present in skeletal, heart, and uterine muscle. In general, these enzymes closely resemble those previously studied from intestinal mucosa (Smith and Bergmann (1)) and from skin, lung, and serum (Fruton (2)). All of these tissues appear to contain mainly enzymes of the type which have been designated exopeptidases; *i.e.*, enzymes which are capable of hydrolyzing peptide bonds adjacent to free terminal amino and carboxyl groups. Endopeptidases which are capable of hydrolyzing peptide linkages which are not adjacent to free terminal amino and carboxyl groups have not been found in significant amounts in these tissues.

Peptidases of Rabbit and Rat Skeletal Muscle

Tables I and II present some of the data obtained with aqueous extracts of rabbit and rat muscle. The hydrolysis of L-leucylglycine (LG) is strongly activated by the presence of 0.001 M $MnSO_4$ and is probably due to the presence of a leucine aminopeptidase similar to those already described in other tissues (1–3). The hydrolysis of the tripeptide L-leucylglycylglycine (LGG) is greater than that of the dipeptide and is also activated by Mn^{++}. The hydrolysis of the tripeptide appears to be caused by the concurrent action of the leucine aminopeptidase, and by a distinct enzyme which is not activated by a metal but which also hydrolyzes diglycylglycine (GGG) and other tripeptides (Smith and Bergmann (1); Fruton (2)). Za-

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mecnik, Stephenson, and Cope (4) have found that extracts of dog muscle hydrolyze LGG.

TABLE I

Proteolytic Enzymes of Rabbit Muscle

The enzyme preparation was a crude aqueous extract. The tests were performed at 40° in a diethylbarbiturate (veronal) buffer at pH 7.8 to 8.0 or in acetate buffer at the acid pH values.

Substrate	Protein N per cc. test solution	pH	Time	Hydrolysis		
				No activator	0.001 M Mn^{++}	0.003 M cysteine
	mg.		*hrs.*	*per cent*	*per cent*	*per cent*
L-Leucylglycine	0.226	8.0	2.5	8	73	
			3.5	12	95	
			6	29	100	
			24	101		
L-Leucylglycylglycine	0.226	7.8	0.5	9	11	
			2.5	33	83	
			3.5	54	98	
			6	92	109	
Diglycylglycine	0.251	8.0	2	20	36	
			24	81	145	
L-Prolylglycine	0.226	7.8	23	7	29	
			51	17	62	
Glycyl-L-proline	0.226	8.0	2		41	
			21	38	103	
			45	65		
Glycyl-L-leucine	0.340	7.8	7	28	26	
			24	51	65	
			48	67	98	
Glycylglycine	0.251	8.0	24	15	86	
Glycyl-L-phenylalanine	0.226	7.9	21	10		
			45	18		
Benzoyl-L-argininamide	0.226	4.0	45	0		
		5.0	26	0		2
		8.0	26	10		8
Carbobenzoxyglycyl-L-phenylalanine	0.226	5.0	26	14		9
		7.9	45	5		
Carbobenzoxy-L-isoglutamine	0.226	5.2	26	9		5
		7.9	45	1		
Carbobenzoxy-L-glutamyl-L-tyrosine	0.226	6.2	45	2		
		5.4	26	4		3
Benzoylglycinamide	0.226	8.0	45	2		
		5.2	45	2		

Prolinase (hydrolysis of L-prolylglycine) and prolidase (hydrolysis of glycyl-L-proline) are also present in muscle extracts, and like the similar enzymes of intestinal mucosa (1) are markedly activated by Mn^{++} ions.

Many other simple dipeptides are hydrolyzed by crude extracts of muscle. The hydrolysis of glycylglycine is strongly activated by Co^{++} ions as well as by Mn^{++}; activation by cobalt appears to be extremely specific for this peptide (5). Berger and Johnson (6) and Maschmann (7) have previ-

TABLE II

Proteolytic Enzymes of Rat Muscle

The experiments were performed with two different crude aqueous extracts of leg muscle (0.186 mg. of protein N per cc.,* and 0.163 mg. of protein N per cc.†) at 40° in diethyl barbiturate buffer at pH 7.8 to 8.0 unless otherwise specified.

Substrate	Time	Hydrolysis		
		No activator	0.001 M Mn^{++}	0.001 M Co^{++}
	hrs.	*per cent*	*per cent*	*per cent*
L-Leucylglycine*	2	3	34	
	4	6	63	
	24	63	101	
L-Leucylglycylglycine*	2	34	59	
	4	62	91	
	24	104	106	
Diglycylglycine*	2	29	25	
	4	49	44	
	5	60	54	
	24	96	99	
L-Prolylglycine*	24	4	32	
	48	10	50	
Glycyl-L-proline*	4	19	26	
	24	54	79	
	48	66	93	
Glycylglycine†	3	19	37	83
	5		53	97
	24	34	96	102
Glycinamide†	48	11	13	9
Benzoylglycine (pH 8.0)†	48	2	0	0
" (" 4.0)†	48	0		
Benzoylglycylglycine†	48	0	1	0
Benzoylglycinamide (pH 8.0)†	24	3		0
" (" 5.0)†	24	3		1
Carbobenzoxyglycyl-L-phenylalanine (pH 8.0)†	48	4		
" (" 5.4)†	48	−1		0‡
Carbobenzoxy-L-isoglutamine (pH 8.0)†	48	3		
" (" 5.4)†	48	0		0‡
Benzoyl-L-argininamide (pH 8.0)†	48	3		
" (" 5.4)†	48	2		3‡

‡ These test solutions contained 0.003 M cysteine.

ously found that the splitting of dipeptides may be specifically activated by different metal ions. The specificity and some other properties of the glycylglycine-splitting enzyme will be reported elsewhere (5).

Most of the enzymes designated as cathepsins which have been found in spleen, liver, and kidney (8) are activated by agents such as cysteine, ascorbic acid, etc. Hydrolysis by muscle extracts of known synthetic substrates for catheptic enzymes has not been found to any significant degree. These include such activities as a carboxypeptidase (substrate, carbobenzoxyglycyl-L-phenylalanine), and various endopeptidases which hydrolyze carbobenzoxy-L-isoglutamine (substrate for papain), benzoyl-L-argininamide (substrate for trypsin and similar enzymes), and carbobenzoxy-L-glutamyl-L-tyrosine (substrate for pepsin and similar enzymes). Fruton (2) has recently found that benzoylglycinamide is hydrolyzed by an enzyme present in human and rabbit sera. This activity has not been detected in muscle extracts. Hippuricase (which hydrolyzes benzoylglycine) is not present in these extracts.

The absence of large amounts of true proteases from our extracts is also indicated by the fact that little or no autolysis could be detected when extracts of the different tissues were incubated at 40° for 48 hours in buffers at pH 4.0 to pH 8.0, and in the presence of cysteine, or various metal ions. Such experiments served as controls for the observation of the hydrolysis of synthetic substrates.

Kies and Schwimmer (9) have described the presence of a cathepsin in calf muscle which has an optimum action near pH 3.5 as judged by the liberation of soluble tyrosine from hemoglobin. Our failure to detect significant amounts of autolysis in our preparations of rat and rabbit muscle may be due to a species difference, or to a much lower concentration of such enzymes in our filtered extracts.

Peptidases of Rabbit Heart and Uterus

Aqueous extracts of rabbit heart muscle and rabbit uterus (Table III) show the same kinds of enzymatic activities as do skeletal muscle extracts. In general, these extracts contain higher peptidase activities than those obtained from the skeletal muscle of this species. Carboxypeptidase and endopeptidase activities have not been detected in the extracts of heart or uterus. This was tested at pH 5.0 and pH 8.0 in the presence or absence of cysteine with carbobenzoxyglycyl-L-phenylalanine, benzoyl-L-argininamide, carbobenzoxy-L-isoglutamine, and carbobenzoxy-L-glutamyl-L-tyrosine.

Peptidases of Human Uterus

Few investigations have been made on the peptidases of human tissues. Berger and Johnson (10) prepared an extract of human duodenum and stud-

TABLE III

Peptidases of Rabbit Heart and Rabbit Uterus

The uterus (fresh weight, 28 gm.) was obtained from a rabbit 2 weeks post partum. The enzyme experiments were performed at 40° in veronal buffer at pH 7.8 to 8.0.

Tissue	Substrate	Protein N per cc. test solution	Time	Hydrolysis		
				No activator	0.001 M Mn^{++}	0.001 M Co^{++}
		mg.	*hrs.*	*per cent*	*per cent*	*per cent*
Heart	L-Leucylglycine	0.0552	2		39	
			5.5	13	84	
			24	87		
	L-Leucylglycylglycine	0.0552	1.5		30	
			2.5	13	51	
			5.5	40	99	
			24	104		
	Diglycylglycine	0.138	1.5	20		
			2	30		
			3	40		
			6.5	74		
			24	96		
	L-Prolylglycine............	0.138	3		14	
			6.5		26	
			24		64	
	Glycyl-L-proline	0.138	3		9	
			6.5		30	
			24		80	
	Glycylglycine	0.063	24	9		77
	Glycyl-L-leucine	0.138	3		13	
			6.5		29	
			24		66	
Uterus	L-Leucylglycine	0.0448	2		27	
			5.5	21	71	
			24	91	101	
	L-Leucylglycylglycine	0.0448	2	20	43	
			5.5	69	94	
	Diglycylglycine	0.224	1.5	71		87
			24	107		149
	L-Prolylglycine	0.224	3	16	17	
			6.5	37	39	
			24	81	84	
	Glycyl-L-proline	0.224	3		34	
			6.5		76	
			24		93	
	Glycylglycine	0.224	24	20		49
			48	28		62
	Glycyl-L-phenylalanine	0.224	24	32	71	30
			48	44	84	44
	Benzoylglycine	0.224	48	0		

TABLE IV

Peptidases of Human Uterine Tissue

The extracts were prepared from two different uteri obtained surgically. The enzymatic experiments were performed in veronal buffer at pH 8.0 to 8.2 and 40°.

Substrate	Protein N per cc. test solution	Time	Hydrolysis		
			No activation	0.001 M Mn^{++}	0.001 M Co^{++}
	mg.	*hrs.*	*per cent*	*per cent*	*per cent*
L-Leucylglycine	0.066	1.5	21	24	
		4	52	50	
		6	66	68	
L-Leucylglycylglycine	0.066	1.5	43	44	
		2	73	72	
		4	96	100	
Diglycylglycine	0.066	1.5	27		
		2	45		
		4	62		
		6	80		
Glycyl-L-proline	0.164	5	11	14	
		24	36	88	
Glycyl-L-phenylalanine	0.164	3		34	
		5		42	
		24		71	
Benzoylglycine (pH 7.8)	0.246	24	1	0	0
" (" 5.0)	0.246	24	1		
L-Leucinamide	0.132	3	16	37	
		4	31	61	
L-Leucylglycylglycine	0.132	0.75	24	26	
		2	61	65	
Diglycylglycine	0.132	0.75	25	12	
		2	53	34	
		2.5	64	43	
		3	75	52	
		4	94	63	
Glycyl-L-leucine	0.132	0.75	38	10	60*
		2	59	22	100*
		3.5	76	38	
Glycylglycine	0.264	0.75	22	48	74
		1.5	41	77	100
		2.75	58	102	101
Glycyl-L-proline	0.396	3.5		74	
Glycylhydroxy-L-proline	0.396	3.5		14	

* This solution contained 0.001 M Zn^{++}.

ied the properties of a leucyl peptidase which was activated by Mg^{++}. Other peptides were also hydrolyzed by this extract. Fruton (2) has studied the peptidases of human skin and found that several enzymes were present, including a leucine aminopeptidase activatable by Mn^{++}, prolidase, and a peptidase which hydrolyzed LGG and GGG.

Table IV presents data obtained with aqueous extracts of human uterus. These extracts contain high peptidase activities similar to those already described in the preceding sections. However, the properties of some of the activities are different from those obtained from other species. The hydrolysis of glycyl-L-leucine by extracts of rabbit tissue is activated by Mn^{++} and Co^{++}. With the human extracts, Mn^{++} and Co^{++} inhibit the hydrolysis of this peptide, but strong activation is produced by Zn^{++}.

The hydrolysis of glycylglycine is activated by Mn^{++}, and to a much greater extent by Co^{++}. This behavior appears to be general for all tissues investigated thus far. As obtained from most tissues, the enzyme is extremely labile. In contrast, the uterine dipeptidase is quite stable at ice box temperatures for many weeks. The properties of these glycylglycine dipeptidases will be described elsewhere (5).

Carboxypeptidase and endopeptidases have not been found in extracts of human uterus when tested under the same conditions described for the other tissues.

Some Observations on Nature of Tissue Peptidases

Leucine Aminopeptidase—Linderstrøm-Lang (11, 12) first pointed out that LG was hydrolyzed by a distinct leucyl peptidase. Berger and Johnson (10, 13) later observed that this enzyme is extremely widely distributed in nature, and that it is strongly activated by Mn^{++} or Mg^{++}. It has since been shown that the enzyme fulfils the specificity requirements of an aminopeptidase (1), and that the activation is apparently a true combination of metal and protein (14). It is of some interest to determine which metal is actually present in the naturally occurring enzyme.

Since Mg^{++} ions form a soluble complex with citrate, whereas Mn^{++} ions do not, it is possible to differentiate between these two metal ions.[1] Various tissue extracts were studied in the presence and absence of 0.01 M sodium citrate without the addition of metal ions (Table V). The hydrolysis of LG and L-leucinamide (LA) was found to be strongly inhibited by citrate ions. It would appear, therefore, that the naturally occurring leucine aminopeptidases are magnesium enzymes. This finding is similar to the observations of McCarty (15) on citrate inhibition of the desoxyribonuclease of beef pancreas.

Leucylglycine-Splitting Enzyme of Human Uterus—Thus far, several different enzymes of animal tissues are known which hydrolyze leucine peptides. These include two types of leucine aminopeptidases, the Mg^{++}- or Mn^{++}-activated enzymes (1, 2, 10), and the cysteine-activated enzymes of beef spleen, beef kidney, and swine kidney (16). These aminopeptidases appear to require similar structural specificity in their substrates, since LG, LGG, and LA are hydrolyzed with velocity constants of the same magnitude. In addition to these enzymes, a peptidase is known which hydrolyzes LGG, GGG, and other tripeptides; this enzyme has little or no action on LG and LA (1).

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[1] This refers to the situation in the neutral pH range. In strongly alkaline solution, citrate will form complexes with Mn^{++} and many other divalent ions.

TABLE V

Effect of Citrate on Tissue Peptidases

The tissue extracts were used without the addition of metal ions.* The tests were performed at 40° in veronal buffer at pH 7.8 to 8.0.

Tissue	Substrate	Protein N per cc. test solution	Time	Hydrolysis	
				No citrate	0.01 M citrate
		mg.	*hrs.*	*per cent*	*per cent*
Rabbit muscle	L-Leucylglycine	0.50	3	55	30
			4.5	94	45
	L-Leucinamide	0.50	2	46	21
			4	83	40
" " (partially purified)	Glycyl-L-proline	0.25	2	17	17
			3	28	26
			5	47	47
			24	102	100
Rabbit heart	L-Leucylglycine	0.126	1.5	16	5
			20	102	20
	L-Leucinamide	0.126	4	41	11
			6	53	13
			24	101	30
" uterus	L-Leucylglycine	0.224	2	26	11
			5	73	27
	L-Prolylglycine	0.224	3	16	21
			6.5	37	32
			24	81	81
	Glycyl-L-proline	0.224	3	26	26
			5	41	40
			24	84	85
Hog intestine (VioBin)	L-Leucylglycine	0.105	1	30	20
			2	67	27
			4	99	43
	L-Leucinamide	0.262	0.75	59	32
			1.5	99	45

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* The activity of these extracts towards the various substrates is from 10 to 100 times greater in the presence of 0.001 M $MnCl_2$.

The experiments reported in Table IV show that uterine extracts contain a tripeptide-splitting enzyme as judged by the hydrolysis of GGG. This hydrolysis is not metal-activated and shows some inhibition by 0.001 M Mn^{++}. Part of the hydrolysis of LGG must also be due to this enzyme. A metal-activated leucine aminopeptidase is present, since the hydrolysis of LA is activated by Mn^{++}.

It has already been demonstrated that the leucine aminopeptidases are magnesium proteins as judged by the inhibition which is produced by citrate. Table VI shows that in a crude extract the hydrolysis of LG and LA is partly inhibited by citrate. The hydrolysis of LGG by the tripeptide-splitting enzyme is apparently too rapid for the small amount of aminopeptidase to influence the over-all rate of hydrolysis, since there is no activation by Mn^{++} or inhibition by citrate.

The hydrolysis of the different substrates by the uterine extract follows the kinetics of a zero order reaction. The proteolytic coefficient, C^0, was calculated from the zero order velocity constant K^0, which is expressed as per cent hydrolysis per minute. In Table V, the coefficient C^0 is equal to K^0/E where E is the enzyme concentration in mg. of protein N per cc. of solution.

It was found that the precipitate obtained with 2 volumes of cold acetone, and then washed with cold acetone and dried, gave a highly active solution when redissolved in water. The relative activity towards the different substrates was changed considerably (Table V). With the acetone-treated preparation, a considerable portion of the tripeptide-splitting enzyme was destroyed, since C^0 for GGG decreased from 3.9 to 0.48 and C^0 for LGG from 5.9 to 1.65. Much of the leucine aminopeptidase activity was retained, since an activating effect of Mn^{++} was apparent both with LGG and LA. However, the activity towards LG was greatly concentrated, since C_{LG} increased about 6-fold from 0.94 to 5.7.

It seems that this activity towards LG must represent a distinct enzyme of a hitherto unknown type. This is suggested by the following evidence. No activation was produced by Mn^{++}, and no inhibition occurred in the presence of citrate. The main action on LG cannot be due to leucine aminopeptidase, since activation was produced by Mn^{++} when the same preparation was tested with LGG or LA. The activity towards LG was not activated by other metals, such as Mg^{++}, Fe^{++}, Co^{++}, or Zn^{++}. Some inhibition was produced by the last two ions. The metal-activated leucine aminopeptidases of hog intestine (1) and of rabbit muscle (unpublished observations) hydrolyze LG and LGG at equal rates. The acetone preparation of uterus hydrolyzed LG about 4 times faster than LGG in the absence of Mn^{++}. Since at least part of the splitting of LGG is due to the concurrent action of the tripeptide-splitting enzyme and the aminopepti-

TABLE VI

Hydrolysis of Leucine Compounds by Crude and Acetone-Treated Extracts of Human Uterus

The tests were performed at 40° and pH 8.2 in veronal buffer. The proteolytic coefficient, C^0, was calculated from the zero order velocity constant, K^0, where $C^0 = K^0/E$. E is expressed as mg. of protein N per cc. of test solution.

Substrate	Protein N per cc. test solution	Time	No addition		Hydrolysis in 0.001 M Mn++	Hydrolysis in 0.01 M citrate
			Hydrolysis	C^0		
Tests performed with crude extract						
	mg.	*hrs.*	*per cent*		*per cent*	*per cent*
L-Leucylglycylglycine	0.111	1	41	6.1	36	40
		1.5	58	5.8	50	62
		2	76	5.7	62	76
L-Leucylglycine	0.222	1.5	17	0.86	18	9
		2.5	31	0.95	32	17
		3.5	44	0.95	43	28
		5	67	0.99	58	41
L-Leucinamide	0.222	2	14	0.53	25	8
		4	23	0.43	50	11
		6	35	0.44	73	16
		24	88		102	38
Diglycylglycine	0.111	0.5	12	3.6		
		1	28	4.2		
		1.5	41	4.1		
		2	51	3.8		
Carbobenzoxy-L-leucylglycine	0.222	24	2			
Carbobenzoxy-L-leucinamide	0.222	24	0			
Tests with acetone-precipitated preparation						
L-Leucylglycylglycine	0.181	1	17	1.6	35	17
		1.5	25	1.5	55	22
		2	36	1.7	76	26
		2.5	49	1.8	83	30
L-Leucylglycine	0.181	0.25	15	5.5	17	16
		0.5	31	5.7	36	30
		0.75	47	5.7	48	46
		1	62	5.7	61	62
		1.5	83		83	84
L-Leucinamide	0.362	0.5	15	1.4	23	15
		1	31	1.5	47	30
		1.5	44	1.4	70	44
		2	60	1.4	85	57
		3	85	1.3	100	86
Diglycylglycine	0.362	1	10	0.46		
		2	21	0.48		
		3	32	0.49		
		4.5	47	0.48		
		5.5	58	0.48		
Carbobenzoxy-L-leucylglycine	0.362	24	1			

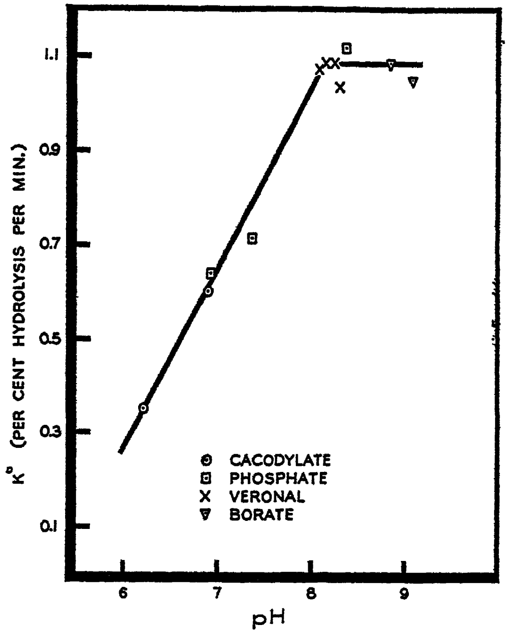

Fig. 1. Hydrolysis of L-leucylglycine as a function of pH. The activities were computed from the zero order velocity constants. The tests were performed at 40° with an acetone-treated enzyme at a concentration of 0.181 mg. of protein N per cc. of test solution. The buffers were at a concentration of 0.04 M, except for the cacodylate which was used at 0.1 M.

Table VII

Effect of Inhibitors on Hydrolysis of L-Leucylglycine

The acetone-treated enzyme was used at a concentration of 0.181 mg. of protein N per cc. of test solution. The experiments were performed at 40° in veronal buffer of pH 8.2.

Time	Hydrolysis				
	No addition	0.1 M fluoride	0.01 M fluoride	0.04 M cyanide	0.003 M cysteine
min.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
15	16		8	14	14
30	32	13	18	28	28
45	48	18	25	41	39
60	63	22	34	51	45

dase, the new enzyme must possess a far greater activity towards LG than it does towards LGG.

The new enzyme does not seem to be an aminopeptidase, since the acetone-treated preparation showed a greater increase of activity towards LG than towards LA in the presence or absence of added Mn^{++}. The presence of a carboxypeptidase can be excluded, since no hydrolysis of carbobenzoxy-L-leucylglycine was observed in 24 hours. It is tentatively suggested that the new enzyme might be a specific dipeptidase. It has already been found that the hydrolysis of glycylglycine by several animal tissues is due to a specific dipeptidase (5), and there is evidence that the splitting of certain other dipeptides may also be due to specific dipeptidases (unpublished observations).

The effect of pH on the hydrolysis of LG by the acetone-treated enzyme is shown in Fig. 1. The enzyme shows a broad zone of maximal activity extending from pH 8.1 to at least pH 9.1. No specific ion effects were observed.

TABLE VIII

Effect of Incubation of Prolidase with $MnSO_4$

The enzyme was obtained from a rabbit muscle extract which had been precipitated with acetone and dried. The concentration was 0.248 mg. of protein N per cc. of test solution. The tests were made at 40° and pH 8.0 in veronal buffer. Incubation of Mn^{++} and enzyme was at 40° and pH 8.0 for 1 hour prior to addition to the substrate (glycyl-L-proline).

Time	Hydrolysis		
	No activator	0.001 M Mn^{++} added without incubation	0.001 M Mn^{++} incubated with enzyme
hrs.	*per cent*	*per cent*	*per cent*
0.5		8	49
0.75			62
1		17	67
2	17	40	92
3	28	63	101
5	47	99	
24	102		

The effect of some inhibitors on the hydrolysis of LG is given in Table VII. The high concentration of sodium cyanide has only a slight effect. This is probably due to its action on the leucine aminopeptidase which is present, since the hydrolysis of LG by this enzyme is known to be strongly inhibited by cyanide (17, 18). Cysteine appears to have a slight initial inhibitory effect which progressively increases. Fluoride in 0.01 M concentration produces a 50 per cent inhibition, while 0.1 M fluoride causes a 63 per cent inhibition.

Prolidase—The hydrolysis of glycyl-L-proline and glycylhydroxy-L-proline by extracts of intestinal mucosa has been ascribed to a distinct peptidase which does not require the presence of peptide hydrogen. This enzyme has been found to be activated by Mn^{++}, but not by other divalent ions (1). In this investigation on other tissues, the prolidases are also specifically activated by Mn^{++} ions. Moreover, no inhibition of activity was observed in the presence of 0.01 M citrate (Table V); so that additional

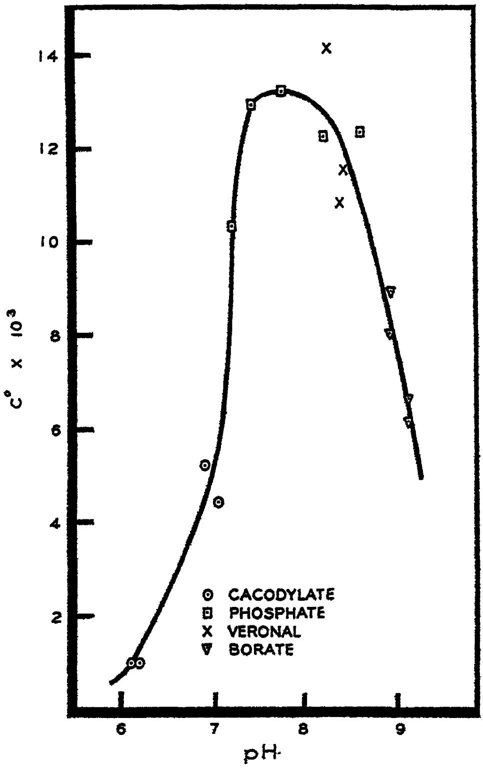

FIG. 2. Activity of prolidase as a function of pH. C^0 is the first order velocity constant per mg. of protein N per cc. The enzyme was obtained from an aqueous extract of rabbit muscle; this was precipitated with 2 volumes of cold acetone and dried. The aqueous extract of the acetone powder was filtered and used for the tests. The enzyme concentration was 0.496 mg. of protein N per cc. of test solution. The enzyme was incubated at 40° with 0.001 M $MnSO_4$ for 2 hours prior to addition to the substrate (glycyl-L-proline). Incubation at pH 8.0 or at the pH of the tests had the same effect on the subsequent rate of hydrolysis of the substrate.

evidence is now available that these enzymes are specific metal proteins in contrast to leucine aminopeptidase which can be activated by either Mn^{++} or Mg^{++}. Likewise, prolinase (hydrolysis of L-prolylglycine), which is activated only by Mn^{++}, is not affected by citrate (Table V).

The activation of prolidase by Mn^{++} is a time reaction (Table VIII).

After incubation of Mn^{++} and the enzyme for 1 hour at 40° and pH 8.0, the hydrolysis proceeds with regular first order kinetics. C, the first order velocity constant per mg. of protein N, is about 10 times greater in the presence of Mn^{++} (0.037) than in the absence of metal ions ($C = 0.0032$). This type of time reaction between a metal and its specific protein, which was first described for leucine aminopeptidase in 1941 (19), has since been found for arginase (20) and phosphatase (21).

The effect of pH on the activity of the prolidase of rabbit muscle is shown in Fig. 2. The enzyme possesses a broad range of maximal activity between pH 7.5 and 8.2.

Although the specificity of prolidase has not yet been elucidated completely, some information is available. Crude and partially purified extracts of hog intestinal mucosa were found to hydrolyze glycyl-L-proline about 8 times faster than glycylhydroxy-L-proline (1). All of the tissues which were examined in this investigation were found to hydrolyze the hydroxyproline peptide as well as the proline peptide. The data in Table IX summarize the results. From the relative constancy of the ratio $C_{GP}:C_{GHP}$, it would appear that these enzymes are homospecific (22). Moreover, the conclusion is probably justified that we are dealing with the activity of a single enzyme from each tissue, since it would be a remarkable coincidence to obtain such constant ratios if more than one enzyme were involved.

TABLE IX

Homospecificity of Prolidases

The enzyme preparation was incubated at 40° in veronal buffer at pH 8.0 together with 0.01 M Mn^{++} for 1 to 2 hours prior to addition to the buffered substrate solution. The final test solutions contained 0.002 M Mn^{++}. The proteolytic coefficient $C = K/E$ where K is the first order velocity constant for the enzyme concentration E expressed in mg. of protein N per cc. of test solution.

Enzyme preparation	C_{GP}	C_{GHP}	Proteolytic quotients, $C_{GP}:C_{GHP}$
Rabbit skeletal muscle (crude)	0.0052	0.00066	7.9
“ “ “ (partially purified)	0.0131	0.00164	8.0
“ heart muscle (crude)	0.0074	0.00088	8.4
Rat skeletal “ “	0.0056	0.00073	7.7
Human uterus	0.0029	0.00032	9.1
“ serum	0.00064*	0.000088*	7.3

* K for 0.5 cc. of serum in 2.5 cc. of enzyme test solution.

Prolidases of intestinal mucosa (1) and of the tissues studied in this investigation do not hydrolyze carbobenzoxyglycyl-L-proline or carbobenzoxyglycylhydroxy-L-proline; a free amino group appears to be required for the action of these enzymes. In preliminary experiments on the purification of rabbit muscle prolidase, it was found that the hydrolysis of glycylglycyl-L-proline paralleled the hydrolysis of diglycylglycine and not that of glycyl-L-proline. It would seem that the imino peptide bond must be adjacent to a free amino group.

DISCUSSION

The classical picture of protein digestion has portrayed an intestinal erepsin as concerned with the terminal stages of the breakdown of peptides to free amino acids. In recent years, it has become apparent that this viewpoint is erroneous in two important aspects. First of all, it is now obvious that the peptidases of intestinal mucosa represent an extremely complex mixture of many different enzymes of exceedingly diverse specificity (1). The term erepsin then becomes a misleading one in so far as it is used to describe a single enzyme or a mixture of a few enzymes. Secondly, evidence has gradually accumulated that the peptidases of intestinal mucosa are not unique. The same or similar enzymes have been found in many other types of animal tissues. Maschmann (7) has found these activities in liver and kidney. Fruton (2) has shown that these enzymes are present in skin, lung, and serum. I has now been demonstrated that similar peptidases are present in skeletal muscle, heart, and uterus. The presence of these enzymes in various tissues throws some doubt on the viewpoint that the enzymes of intestinal mucosa are solely concerned with digestion. It is more likely that all of these enzymes are intracellular in nature and concerned with similar functions, regardless of the tissue in which they are found.

EXPERIMENTAL

The enzyme experiments were performed in 2.5 cc. volumetric flasks at a temperature of 40°. Hydrolysis was measured on 0.2 cc. samples by the titration method of Grassmann and Heyde (23). The substrates were present in a concentration of 0.05 M. Hydrolysis of one peptide bond is expressed as 100 per cent. Controls were performed by incubation of the tissue extracts in the absence of substrate. No significant autolysis was detected.

The extracts were prepared by washing the tissues with cold water to remove as much blood as possible. The tissues were then minced and homogenized with cold distilled water in a Waring blendor. The extracts were adjusted to between pH 7.2 and 7.5 (phenol red) and centrifuged or filtered. No differences in activity were found when the extracts were prepared in physiological saline or veronal buffer (pH 7.8), except that more inactive protein was dissolved.

The technical assistance of Rosalind Pack, Douglas M. Brown, and Marie S. Hanson is gratefully acknowledged. Thanks are also due to Dr. Emil Holmstrom of the Department of Obstetrics and Gynecology for the uteri used in this investigation.

SUMMARY

1. Extracts of rabbit muscle, heart, and uterus as well as those of rat muscle and human uterus contain high peptidase activities which can hydrolyze a variety of simple peptides. Leucine aminopeptidase, prolinase, prolidase, and a tripeptide-splitting enzyme have been found in all of these tissues. Endopeptidases that hydrolyze peptide linkages, which are not adjacent to free terminal amino or carboxyl groups, have not been found to any significant extent. Carboxypeptidase has not been detected in these extracts.

2. The metal-activated leucine aminopeptidases of various tissues are strongly inhibited by citrate ions, indicating that these enzymes are magnesium protein compounds.

3. Some evidence is presented for a new type of enzyme in human uterus which hydrolyzes L-leucylglycine, and which has at best a very much weaker action on L-leucylglycylglycine and L-leucinamide. This suggests that an aminopeptidase is not responsible for the action. Blocking of the amino group (carbobenzoxy-L-leucylglycine) prevents the hydrolysis; hence the action of a carboxypeptidase can be excluded. The new enzyme may be a specific dipeptidase.

4. The prolidases of various tissues and human serum are homospecific manganese enzymes which hydrolyze glycyl-L-proline and glycylhydroxy-L-proline with the same relative effectiveness.

5. The concept of an intestinal erepsin is outmoded and misleading, since the same or similar enzymes have been found in many other types of tissues.

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THE GLYCYLGLYCINE DIPEPTIDASES OF SKELETAL MUSCLE AND HUMAN UTERUS*

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Until recent years, it was generally assumed that the hydrolysis of all simple dipeptides was due to a single enzyme, dipeptidase. In 1929, Linderstrøm-Lang (1) demonstrated that the two dipeptides, leucylglycine and alanylglycine, were split by different enzymes. One of these has since been found to be a leucine aminopeptidase which hydrolyzes a variety of leucine peptides including leucinamide (2). Because of this and similar studies, the existence of a dipeptidase which is capable of hydrolyzing many kinds of dipeptides has become doubtful. The studies of Bergmann and his collaborators (3, 4), have amply demonstrated that proteolytic enzymes require for their action the presence of specific amino acid residues in the substrates as well as the presence or absence of amino and carboxyl groups.

It has now been found that the enzyme of animal tissues which hydrolyzes glycylglycine appears to fulfil the specificity requirements of a dipeptidase, but in a sense quite different from that in which the term was used earlier. Substitution of amino or carboxyl groups renders the peptide resistant to hydrolysis. Moreover, the activity towards glycylglycine does not appear to parallel the hydrolysis of any other dipeptide which has yet been studied. It appears justifiable to ascribe the enzyme action to a specific glycylglycine dipeptidase.

Properties of Glycylglycine Dipeptidase of Rat Muscle

Specificity—Table I presents the data obtained with a crude fresh aqueous extract of rat skeletal muscle. The splitting of glycylglycine (GG) is strongly activated by Co^{++} (5) and to a lesser degree by Mn^{++}. Mg^{++} does not accelerate this hydrolysis, and Zn^{++} acts as an inhibitor. These extracts do not hydrolyze compounds in which the free amino group is suppressed as in carbobenzoxyglycylglycine or benzoylglycylglycine, or in which both the amino and carboxyl groups are substituted as in carbobenzoxyglycylglycinamide and benzoylglycinamide. No hydrolysis of hippuric acid (benzoylglycine) was detected in these extracts; hippuricase is obviously a different enzyme from the dipeptidase.

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* This investigation was aided by a grant from the United States Public Health Service.

The hydrolysis of diglycylglycine (GGG) by crude tissue extracts is due to a distinct enzyme from the dipeptidase. This is shown by the fact that the initial hydrolysis of the tripeptide is only slightly activated by metal ions, and that, in the absence of metal ions, the second peptide bond is split extremely slowly. In the presence of Mn^{++} or Co^{++}, the second peptide bond is hydrolyzed quite rapidly (Table II). The tripeptide-splitting enzyme is much more stable than the dipeptidase. Under conditions in which most of the dipeptidase activity is lost, *e.g.* standing at pH 7.6 and 40° for 5 hours, the hydrolysis of the tripeptide is relatively unimpaired. Under the same conditions, most of the capacity of the extract to hydrolyze glycylglycinamide and glycinamide is unaffected. However, since there is some loss of activity towards these substrates, it would appear that the dipeptidase may be able to split the two amides extremely slowly.

TABLE I

Action of Rat Skeletal Muscle Extracts on Glycine Compounds

The tests were performed at 40° in veronal buffer at pH 8.0. The enzyme preparation was a freshly prepared crude extract of rat skeletal muscle.

Substrate	Protein N per cc. test solution	Time	Hydrolysis		
			No activator	0.001 M Mn^{++}	0.001 M Co^{++}
	mg.	*hrs.*	*per cent*	*per cent*	*per cent*
Glycylglycine	0.158	3	19	37	83
		24	34	96	102
Benzoylglycinamide	0.316	22	1		0
Benzoylglycine	0.316	22	0	0	2
Carbobenzoxyglycylglycine	0.316	22	0		0
Benzoylglycylglycine	0.474	45	4		1
Glycinamide	0.474	22	3		8
Glycylglycinamide	0.316	22	20		20
Carbobenzoxyglycylglycinamide	0.316	22			5

The hydrolysis of GGG, L-leucylglycylglycine, and other tripeptides has been ascribed to a distinct enzyme. Although this enzyme has not yet been obtained in homogeneous form, highly active preparations from intestinal mucosa possess only slight activity towards most dipeptides (2).

The enzyme which hydrolyzes GGG may be activated to some extent by Co^{++}. The enzyme preparations which had been heated at 40° for 5 or 12 hours show little hydrolysis of glycylglycine in 4 hours; yet there is a greater effect of Co^{++} on the splitting of the tripeptide than can be due to the action of the dipeptidase.

Other glycine-containing peptides are hydrolyzed by a crude extract of rat muscle (6), but these appear to be unrelated to the hydrolysis of GG.

Since further studies are in progress on some of these enzymes, only a few salient observations which differentiate some of these activities from GG dipeptidase will be given. Unlike the GG-splitting enzyme, which is quite labile, the enzyme in rat muscle which hydrolyzes glycyl-L-leucine is much more stable at ice box temperatures. Moreover, this enzyme is not activated by Co^{++}. The hydrolysis of glycyl-L-phenylalanine is not acti-

TABLE II

Differentiation of Glycylglycine Dipeptidase from Tripeptide-Splitting Enzyme

The tests were performed at 40° in veronal buffer at pH 7.8 with a fresh extract of rat muscle. The second series was performed with the same extract after it had been incubated at 40° for 5 hours and filtered. The third series was made with the same extract after heating for 12 hours at 40°.

Substrate	Protein N per cc. test solution	Time	Hydrolysis		
			No activator	0.001 M Mn^{++}	0.001 M Co^{++}
	mg.	*hrs.*	*per cent*	*per cent*	*per cent*
Glycylglycine	0.185	1	3	17	31
		2	2	28	62
		4	7	51	100
Diglycylglycine	0.185	1	15	16	20
		2	31	25	49
		4	55	47	107
		20	102	136	191
Glycylglycinamide	0.370	24	24	25	24
Glycinamide	0.370	24	11	6	15
Tests with same enzyme after 5 hrs. at 40°					
Glycylglycine	0.107	4	2	3	3
		24	11	12	23
Diglycylglycine	0.107	2	26	17	36
		4	46	37	66
		6	64	53	87
		24	102	105	113
Glycylglycinamide	0.214	24	12	12	11
Glycinamide	0.214	24	6	5	8
Tests with same preparation after 12 hrs. at 40°					
Glycylglycine	0.079	4	1	1	5
		24	8	10	19
Diglycylglycine	0.158	2	21	18	33
		4	40	33	67
		5.5	54	48	79
		24	98	96	99

vated by Co++, but is activated by Mn++ and to a much greater degree by Mg++. The enzyme which hydrolyzes glycyl-L-alanine is not affected by Co++. Leucine aminopeptidase, prolidase, and prolinase have all been obtained free of the GG dipeptidase.

Product of Hydrolysis—Since the action of the dipeptidase appears to be so specific, it was desirable to isolate the compound formed after the action of the tissue extracts. The expected amount of glycine was isolated as the carbobenzoxy compound. The details are given in the experimental section.

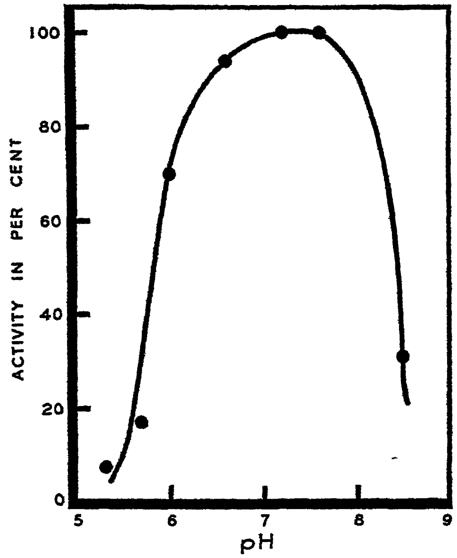

FIG. 1. Stability of glycylglycine dipeptidase as a function of pH as determined after incubation at 40° for 2.5 hours. Residual activity of the enzyme was then determined in veronal buffer at pH 8.0 in the presence of 0.001 M Co++. The enzyme preparation was a crude extract of rat muscle.

Stability—Fig. 1 shows the results of an experiment in which a fresh extract of rat skeletal muscle was adjusted to a series of different pH values with requisite amounts of 0.1 M NaOH or HCl. After incubation at 40° for 2.5 hours, the solutions were readjusted to pH 8.0. The specific enzyme activities as determined from the zero order velocity constants are plotted in Fig. 1 as a percentage of the maximal activity which was observed.

The dipeptidase is obviously an extremely labile enzyme and even at the region of maximal stability, about pH 7.5, about 50 per cent of its activity may be lost on standing at ice box temperatures overnight. The presence of Co++ or Mn++ in optimal amounts (0.001 M) improves the stability somewhat.

Kinetics; Effect of Enzyme, pH, and Cobalt Concentration—The hydrolysis of GG follows zero order kinetics under the conditions which we have used, provided that the measurements are made within a few hours so that the inactivation of the enzyme may be considered to be negligible. In fact, the enzyme is greatly stabilized by the presence of its substrate. Fig. 2 shows that the amount of hydrolysis is proportional to the enzyme concentration over a reasonable range. The values obtained at the two lowest enzyme concentrations are somewhat low. This is probably due to a partial inactivation of the enzyme over the period of 5 to 6 hours necessary for adequate measurements of hydrolysis. At the higher enzyme concentrations, satisfactory velocity constants could be obtained within 1 to 2 hours.

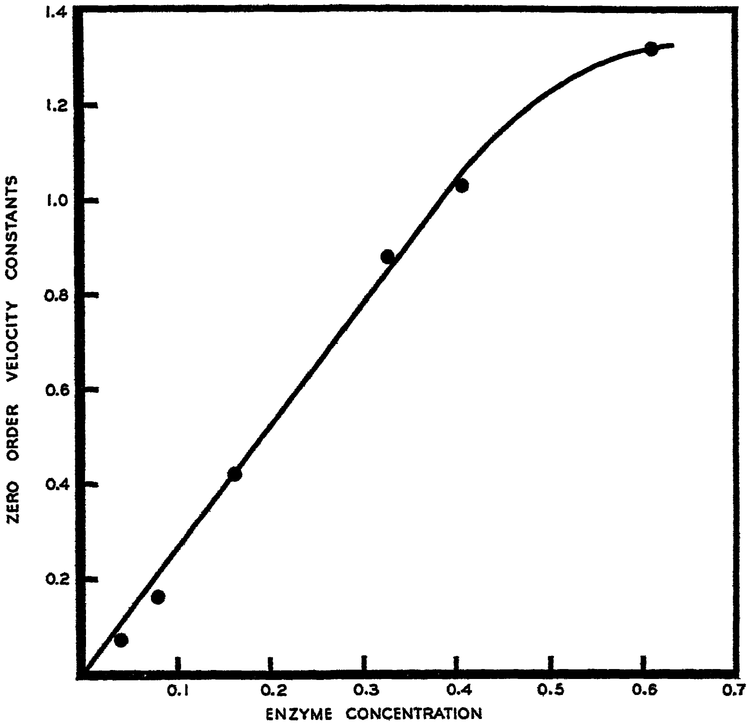

Fig. 2. Activity as a function of enzyme concentration (in mg. of protein N per cc.) for an extract of rat skeletal muscle. The zero order velocity constants are in per cent hydrolysis per minute. The experiments were performed at 40° in veronal buffer at pH 8.0 in the presence of 0.001 M Co^{++}.

The presence of 0.001 M Co^{++} ions is sufficient to produce maximal activation of the enzyme. Because of the lability of the enzyme, the effect of different Co^{++} concentrations has to be determined during a short time with a high enzyme concentration. The data are shown in Fig. 3 where the zero order velocity constants are presented as a function of log Co^{++} concentration. K_d, the dissociation constant of a hypothetical enzyme-metal compound, is equal to 2.8×10^{-5} M. The velocity constant obtained without the addition of metal ions is also indicated in Fig. 3. The maximal activation produced was 10 times that found in the absence of Co^{++}.

Effect of Cysteine—When metal ions are not added to the tissue extracts, the hydrolysis of GG is strongly inhibited by the presence of 0.003 M cysteine. This inhibition, which can be partly reversed by the addition of Mn^{++} or Co^{++}, may be interpreted as a binding of metal ions by cysteine in competition with the protein. The formation of cysteine complexes with cobalt and other heavy metals is well known (7). The GG dipeptidase is not related to the cysteine-activated cathepsins or to the bacterial enzymes whose activity is enhanced by the presence of both cysteine and metal ions (Maschmann (8)).

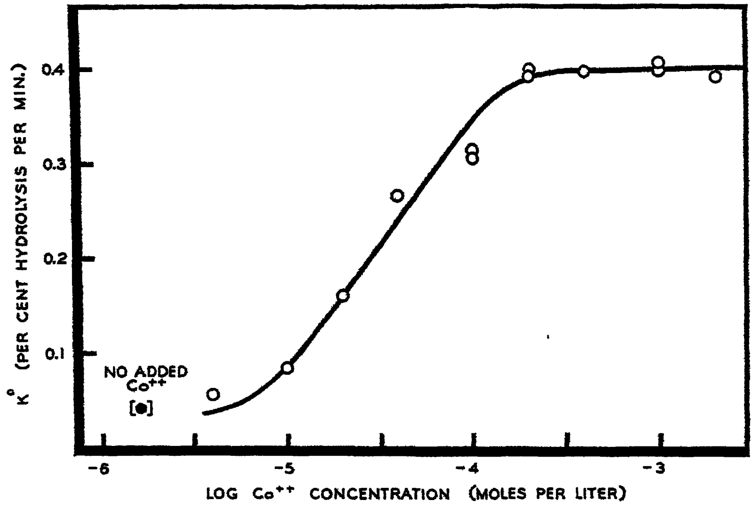

FIG. 3. Activity (zero order velocity constants, K^0) as a function of Co^{++} concentration for the splitting of glycylglycine by rat muscle. The solid point represents the activity in the absence of added metal ions. K_d, the dissociation constant, is 2.8×10^{-5} M.

Glycylglycine Dipeptidase of Human Uterus and Other Tissues

The splitting of GG is performed by many types of tissues, and, in all cases, is strongly activated by Co^{++} (5, 9). Maschmann (5) used, among other sources, liver, kidney, and intestinal mucosa of rabbit, guinea pig, and mouse. These findings suggest that the specific dipeptidase is widely distributed in tissues. However, no specificity studies seem to have been performed.

Human uterine tissue has proved to be an extremely rich source of peptidases, and particularly of the GG-splitting enzyme (6). In contrast to the enzyme found in most tissues, the uterine enzyme was found to be stable for many weeks at ice box temperatures, and this proved to be an extremely useful property.

TABLE III

Hydrolysis of Glycine Compounds by Tissue Extracts

These experiments were performed at 40° in veronal buffer at pH 8.0.

Tissue	Substrate	Protein N per cc. test solution	Time	Hydrolysis		
				No activator	0.001 M Mn^{++}	0.001 M Co^{++}
		mg.	*hrs.*	*per cent*	*per cent*	*per cent*
Human uterus	Glycylglycine	0.264	0.75	22	54	70
			2	55	98	100
	Diglycylglycine	0.132	0.75	25	12	24
			2	53	34	100
			3	75	52	
			4	98	63	
	Benzoylglycylglycine	0.264	24	0		0
	Benzoylglycine	0.264	24	1		
	Benzoylglycinamide	0.264	24	1		0
	Glycinamide	0.264	24	6		6
	Glycylglycinamide	0.264	24	14		10
	Carbobenzoxyglycylglycinamide	0.264	24	2		1
Rabbit skeletal muscle	Glycylglycine	0.251	2	5	18	15
			24	15	86	82
	Diglycylglycine	0.251	2	20	36	
			24	81	145	
Rabbit uterus	Glycylglycine	0.224	24	20		49
" heart muscle	"	0.063	2	2		19
			24	7		77
Rabbit serum	"	0.2 cc. in 0.5 cc.	7	8		22
			24	14		52
Hog intestine	"	0.80	1	12		28
			4	37		103
Human serum	"	0.5 cc. in 2.5 cc.	48	18	46	80

Table III shows the data obtained on the hydrolysis of various glycine-containing compounds. The specificity appears to be identical with the homologous enzyme of rat muscle. It should be noted that Mn^{++} inhibits the splitting of GGG. The initial rate of hydrolysis of the tripeptide is not accelerated by Co^{++}, but the later hydrolysis is markedly increased. As in the case of the enzyme from rat muscle, the hydrolysis of the first peptide bond of the tripeptide is due to a distinct enzyme. In the uterine extracts, the Co^{++}-activated dipeptidase is more active than the tripeptide-splitting enzyme. It is obvious that as fast as GG is liberated from the tripeptide it will be split by the dipeptidase and thus produce an apparent activation by

TABLE IV

Effect of Enzyme Concentration on Glycylglycine Hydrolysis

The enzyme preparation was an aqueous extract of acetone-dried powder from human uterus. The tests were performed in the presence of 0.001 M $CoCl_2$ at 40°. The solutions were buffered at pH 8.1 with veronal. K^0 is given as per cent hydrolysis per minute. C^0 is K^0 per mg. of protein N per cc.

Protein N	Time	Hydrolysis	K^0	C^0	C^0, average
mg. per cc.	*hrs.*	*per cent*			
0.00452	4	15	0.063	13.9	
	5	17	0.057	12.6	
	6	22	0.061	13.5	
	7	26	0.062	13.7	13.4
0.00904	3.5	24	0.114	12.6	
	4.5	33	0.122	13.5	
	5.5	39	0.118	13.1	
	6	45	0.125	13.8	
	7	50	0.119	13.2	13.2
0.0181	1.5	22	0.244	13.5	
	2	29	0.242	13.4	
	2.5	36	0.240	13.3	
	3	42	0.233	12.9	13.3
0.0362	1	29	0.48	13.3	
	1.5	45	0.50	13.8	
	2	60	0.50	13.8	
	2.5	71	0.47	13.0	
	3	83	0.46	12.7	13.3
0.0724	0.25	14	0.93	12.8	
	0.5	28	0.93	12.8	
	0.75	50	1.11	15.6	
	1	59	0.98	13.5	
	1.25	73	0.97	13.4	13.6
0.109	0.25	23	1.53	14.0	
	0.5	49	1.63	15.0	
	0.75	69	1.53	14.0	
	1	84	1.40	12.8	14.0

Co^{++}. The splitting of glycinamide and glycylglycinamide is not accelerated by Co ions.

Table III also shows the activating effect of Co^{++} on the hydrolysis of GG by several other tissues and by human and rabbit sera. Extracts of rabbit skeletal muscle showed the same specificity with various glycine derivatives as that already presented for rat muscle and human uterus.

Kinetics of Uterine Enzyme—The hydrolysis of GG by the uterine dipeptidase is proportional to the enzyme concentration over a tested range of 1 to 24 times (Table IV), and the hydrolysis follows zero order kinetics. The experiments were performed by the addition of enzyme to the solution containing the buffered substrate in the presence of 0.001 M Co^{++}. No prior incubation of enzyme and metal was found to be necessary, as the enzyme showed its full activity instantaneously. This is in contrast to the behavior of leucine aminopeptidase (2) and prolidase (6) in which the reaction of enzyme and metal takes considerable time.

TABLE V

Metal Activation and Inhibition of Glycylglycine Splitting by Human Uterine Extract

Each test sample contained 0.264 mg. of protein N per cc. of the crude extract. The tests were performed at 40° in veronal buffer at pH 8.0. The metal ions were present in 0.001 M concentration.

Substance tested	Hydrolysis			
	45 min.	90 min.	120 min.	165 min.
	per cent	*per cent*	*per cent*	*per cent*
Co^{++}	74	100	101	
Mn^{++}	54	77	98	104
Mg^{++}	21	43	55	64
Zn^{++}	4	6	7	10
None	22	41	55	69
Cysteine (0.03 M)	10	20	29	45
" + Co^{++}	24	43	54	64
" + Mn^{++}	17	35	52	77
Citrate (0.01 M)	26	47	59	73

The precipitate obtained by adding 2 volumes of cold acetone to a crude filtered extract of uterus could be collected and washed with acetone. A filtered aqueous extract of the acetone-dried powder was about twice as active per mg. of protein N as the original extract. The experiments in Table IV were performed with an aqueous extract of the acetone powder.

The acetone-dried powder has been found to be a rich source of many of the peptidase activities of human uterus, and these preparations promise to be extremely useful in purification studies. About 80 per cent of the activity towards GGG is destroyed by the acetone treatment, while the dipeptidase activity is concentrated about 2-fold. This furnishes additional proof that the dipeptide and tripeptide are hydrolyzed by distinct enzymes.

pH Activity Function of Uterine Dipeptidase—In Fig. 4 is shown the activity of the GG dipeptidase as a function of pH. The enzyme is active over a narrow range and shows a sharp maximum near pH 7.6. The tests were performed with the acetone-precipitated enzyme. Hydrolysis was followed until 50 to 60 per cent splitting had occurred. The pH was then determined and found to differ by less than 0.1 unit from the initial values. The pH measurements were made with a glass electrode at room temperature.

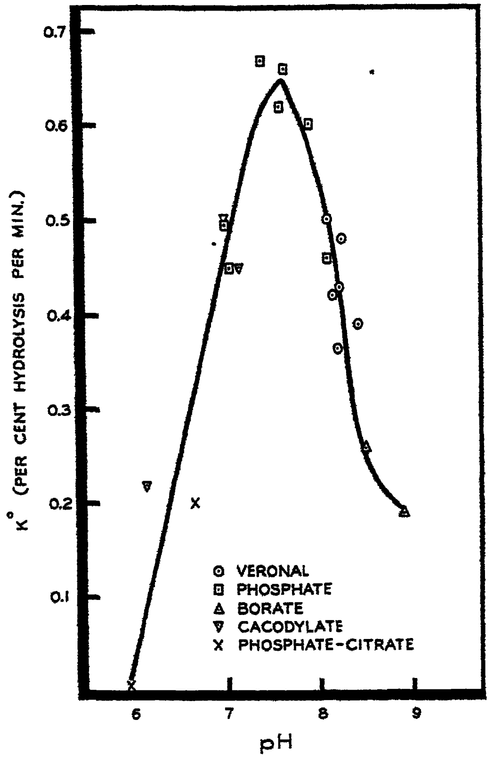

FIG. 4. Activity as a function of pH of the glycylglycine dipeptidase of human uterus. The buffers used were veronal (0.04 M), phosphate (0.04 M), borate (0.04 M), phosphate-citrate (0.08 M), and cacodylate (0.1 M). The temperature was 40°. The enzyme was an acetone-precipitated preparation, and was used at a concentration of 0.0362 mg. of protein N per cc. of test solution. Co^{++} was present at 0.001 M.

Effect of Metals, Cysteine, and Citrate—In Table V are presented the data showing the effect of various metals ions on the hydrolysis of GG by human uterus. Mg^{++} is without any action, and Zn^{++} is a powerful inhibitor of the enzyme. Cysteine is also an inhibitor, but this effect may be prevented by Co^{++} or Mn^{++}. Citrate is without action on the enzyme.

DISCUSSION

It is difficult to relate our study of the GG dipeptidase to many of the earlier investigations on "dipeptidase" because of the variety of peptides which were then used and which were assumed to be hydrolyzed by one and the same enzyme from many different tissues (10). The data presented in this paper strongly suggest that GG is hydrolyzed by an extremely specific dipeptidase which has little or no action on derivatives of the dipeptide in which the amino or carboxyl groups are substituted. It is also well known from earlier observations (11, 12) that glycylsarcosine is not hydrolyzed by crude extracts of tissues which act on GG. The peptide hydrogen is, therefore, essential for the action of the dipeptidase. The enzyme would appear to require three points of attachment to its substrate: through the amino group, the carboxyl group, and the peptide hydrogen.

While the nature of the enzyme-substrate combination is unknown, one observation is suggestive in this regard. When glycylglycine is incubated with Co^{++} ions at pH 8.0, a pink color develops which is much stronger than that given by Co^{++} alone. Fig. 5 shows the absorption spectrum of 0.01 M $CoCl_2$ at pH 7.8 in the presence of 0.125 M GG, GGG, and glycine after the mixtures had been allowed to stand for 24 hours at room temperature. The simplest interpretation of this phenomenon is that a specific coordination compound is formed at this pH. The tendency of Co^{++} to form complex amino compounds is, of course, well known.

The specific combination of GG and Co^{++} ions leads to the idea that the function of the metal is to act as a bridge in forming the enzyme-substrate compound. The specificity of the enzyme would depend, therefore, not only on the protein, but also on the ability of the metal ion to combine with the substrate. Further studies are now in progress on specific coordination compounds of peptides with metal ions.

EXPERIMENTAL

The crude extracts were prepared by homogenizing the tissues in a Waring blendor. The preparation was adjusted to pH 7.5 (phenol red) and the insoluble residue removed by centrifugation or filtration. Since the dipeptidase of rat muscle was unstable, the extracts were prepared in the cold and used within 1 to 2 hours after the death of the animal. The observations on extracts of human uterine tissue were generally made within a few days.

Hydrolysis was measured on 0.2 cc. samples by the method of Grassmann and Heyde (13). The enzyme experiments were performed in 2.5 cc. volumetric flasks and at a substrate concentration of 0.05 M. Hydrolysis is expressed as 100 per cent for the complete splitting of one peptide bond. Appropriate controls were performed by incubation of the tissue extracts or substrates under the conditions used for the enzyme experiments.

The substrates used in this investigation were prepared as described in the bibliographic citations: glycylglycine (14), diglycylglycine (14), benzoylglycylglycine (14), glycinamide hydrochloride (15), benzoylglycinamide (15), glycylglycinamide acetate (16), carbobenzoxyglycylglycine (17), and carbobenzoxyglycylglycinamide (16). Analytical purity was checked by nitrogen content and by melting point when applicable.

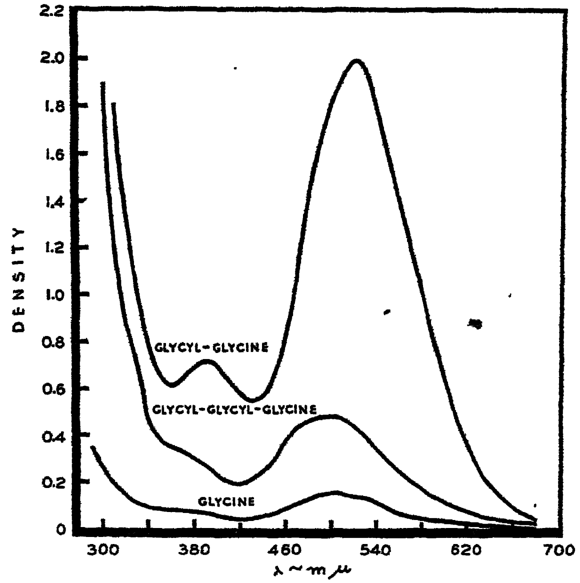

FIG. 5. Optical density of $CoCl_2$ (0.01 M) in the presence of glycylglycine, diglycylglycine, and glycine at a concentration of 0.125 M. The reaction was allowed to proceed for 24 hours at room temperature (about 30°). The measurements were made with a Beckman ultraviolet spectrophotometer and a 1 cm. quartz cell.

Isolation of Carbobenzoxyglycine from Hydrolysis of Glycylglycine—330 mg. of glycylglycine were dissolved in water and brought to pH 8 by the addition of NaOH. For the total volume of 50 cc., an extract of rat skeletal muscle was present at a concentration of 0.28 mg. of protein N per cc., and Co^{++} ions at 0.001 M. When the titration samples showed that 100 per cent splitting had occurred, 1 cc. of concentrated HCl was added, and the mixture was heated in a boiling water bath for 15 minutes. The solution was cooled, filtered with the aid of analytical Celite, and concentrated *in vacuo* to a volume of about 10 cc. The solution was made alkaline to litmus, and 0.8 cc. of carbobenzoxy chloride was added at 0°. On acidification to Congo red, 620 mg. of prisms were obtained. After recrystallization from chloroform the melting point was 120–121°. The mixed melting point with carbobenzoxyglycine was 120–121°.

$C_{10}H_{11}O_4N$ (209.2). Calculated, N 6.70; found, N 6.83

The technical assistance of Rosalind Pack and Marie S. Hanson is gratefully acknowledged.

SUMMARY

1. The hydrolysis of glycylglycine by animal tissue extracts is due to a specific dipeptidase which is strongly activated by Co^{++} and to a lesser extent by Mn^{++} ions. Hydrolysis may be prevented by substitution of the amino group (benzoylglycylglycine), the carboxyl group (glycylglycinamide), or both (carbobenzoxyglycylglycinamide).

2. The glycylglycine dipeptidase of rat muscle is extremely labile and shows maximal stability at pH 7.2 to 7.6. The apparent dissociation constant of the cobalt-enzyme compound is 2.8×10^{-5} M. Hydrolysis follows zero order kinetics under the conditions used.

3. The enzyme is also present in extracts of rabbit skeletal, heart, and uterine tissue and in human uterus. The enzyme of human uterus is stable at low temperatures and it may be precipitated with acetone and dried. The hydrolysis of glycylglycine by this enzyme also follows zero order kinetics and its activity is proportional to the concentration over a wide range. Its maximal activity is at pH 7.6.

4. Glycylglycine forms a specific coordination compound with Co^{++} as is shown by an intensification of the Co^{++} spectrum. It is suggested that the formation of such a compound may be significant for the specificity of the enzyme.

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THE CHARACTERIZATION OF PURINES AND PYRIMIDINES BY THE METHOD OF COUNTER-CURRENT DISTRIBUTION*

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Adequate criteria are not available for precise characterization of purines and pyrimidines. These compounds do not possess melting points suitable for precise characterization, do not readily form simple derivatives except salts, and nitrogen analyses are never adequate to detect the presence of small amounts of similar impurities. Even their characteristic ultraviolet absorption spectra are generally unsatisfactory owing to similarities of spectra and difficulties in the detection of small contaminations of one by another. Recently Vischer and Chargaff (1) have begun to apply unidimensional paper chromatography to the qualitative characterization of very small samples of adenine, guanine, and xanthine.

The admirable counter-current distribution technique developed by Craig (2, 3) has been applied to this problem and has proved to be a convenient and precise method for qualitative and quantitative characterization of individual compounds and simple mixtures. Estimation of the substances present in the series of tubes resulting from the distribution may be readily made by measurement of the partial absorption spectra. The partition coefficients (K) found for a series of purines and pyrimidines and related compounds determined in a system consisting of mutually saturated *n*-butanol and 1 M potassium phosphate buffer of pH 6.5 are given in Tables I and II.

The conditions described are particularly advantageous for the separation of adenine and guanine. The homogeneities (4) of samples of these purines, isolated in the course of certain metabolic experiments (5), have been demonstrated to within 1 or 2 per cent. The presence of small amounts of adenine in commercial samples of hypoxanthine and of guanine in commercial adenine has been readily demonstrated, and routine characterizations have been made of compounds and mixtures encountered in the course of certain synthetic work.

Although the separation of mixtures of the nucleotides would require an impractical number of transfers, they are readily separated from the nucleosides and from the free bases, and this offers an excellent means for rapid

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analysis of the products of hydrolysis of the nucleotides. For instance, the hydrolysis of cytidylic acid in 6 N HCl at 135° for 1 hour yields a mixture of unchanged cytidylic acid, cytidine, and cytosine.

TABLE I

Partition Coefficients in n-Butanol and 1 M Phosphate, pH 6.5, System

		Partition coefficient*	Concentration in Tube 0	Absorption maxima
			mg. per cc.	*mμ*
Purines	Adenine	2.77†	2.65	260
		2.14†	0.12	
	Guanine	0.45	0.11	247, 274
	Hypoxanthine	0.54	2.00	250
	Xanthine	0.46	0.15	269
	Isoguanine‡	0.28	0.56	239, 286
	Uric acid	0.11	Saturated	240, 293
	2-Thioadenine‡	0.48	0.8	228, 256–262, shoulder at 283
	2,6-Diaminopurine‡	1.21	0.46	250, 281
Pyrimidines	Thymine	1.11	2.00	265
	Uracil	0.401	2.00	260
		0.400	0.56	
	Cytosine	0.207	2.00	267
		0.206	0.50	
	Uramil	0.029	Saturated	End-absorption
	Barbituric acid	0.069	"	275
	4,5,6-Triaminopyrimidine	0.490	0.25	278
	4,6-Diamino-5-formamidopyrimidine	0.16§	0.30	260
	4,6-Diamino-5-thioformamidopyrimidine	0.96	0.5	260
	4,6-Diamino-5-benzeneazopyrimidine	6.12	0.16	246
	2,4,5,6-Tetraminopyrimidine	0.048	0.48	275

* Calculated from twelve or twenty-four transfer distributions.

† Variations in the constant for adenine are not directly proportional to concentration but are perhaps associated with the purity of the butanol utilized.

‡ Prepared by Dr. Aaron Bendich.

§ This figure was erroneously given (5) as 0.225.

Under conditions that are the basis for a quantitative estimation of purines (6) (1 N HCl at 100° for 1 hour) adenylic acid was demonstrated to be completely hydrolyzed to adenine. Under these conditions the hydrolysis of yeast (pentose) nucleic acid yielded only the purines, adenine (concentration maximum in Tube 18 out of twenty-four) and guanine (Tube 7), and pyrimidine nucleotides (Tubes 0 and 1). Thymus (desoxypentose) nucleic acid led to a similar distribution pattern with the addition of a trace of material of $K = 1.0 \pm 0.1$, probably thymine. The materials in Tubes 0 and 1 in this distribution (apparent $K = 0.014$, $\lambda_{\text{max.}} = 267$) were undoubtedly the pyrimidine desoxyribotides, individual samples of which were not available for determination of constants. The work with paper chromatography (1) has also detected no purines other than adenine and guanine in nucleic acid hydrolysates. The more vigorous hydrolysis conditions necessary to split the pyrimidine nucleotides have been shown to result in extensive decomposition of the free purines, and application of the technique for further characterization of nucleic acids must await studies of the hydrolysis of nucleic acids and their components.

TABLE II

		Partition coefficient
Nucleosides	Adenosine	0.76
	Guanosine	0.12
	Inosine	0.12
	Xanthosine	0.05
	Uridine	0.12
	Cytidine	0.08
Nucleotides	Adenylic acid	0.02_1
	Guanylic "	0.02_0
	Uridylic "	0.01_5
	Cytidylic "	0.01_1
Miscellaneous	Picric acid	9.2
	Brucine	1.3

EXPERIMENTAL

Approximately 0.2 to 2.0 mg. per cc. of the compound or mixture to be distributed was dissolved in butanol-saturated 1 M phosphate buffer of pH 6.50, or if the mixture were a solution, the pH was adjusted to 6.5 and the buffer concentration was adjusted to approximately 1 M. 8 cc. of the solution were introduced into the first compartment (Tube 0) of a twenty-five compartment distribution machine (2). The distribution (2) was usually carried to twenty-four transfers, although in the case of mixtures of adenine and guanine twelve transfers are sufficient, while in the case of uracil and cytosine 56 transfers are needed to obtain a reliable characterization of both components. Typical distribution curves are illustrated in Figs. 1

and 2. When the distribution was completed, the contents of the compartments were siphoned into separate test-tubes. From each of the tubes a homogeneous mixture of 1 cc. of each phase and 3 cc. of 45 per cent aqueous ethanol was prepared. With initial concentrations in the range of 0.2 to 1.0 mg. per cc. this dilution gives appropriate concentrations for direct measurement of the optical density in a 1 cm. cell in the Beckman model

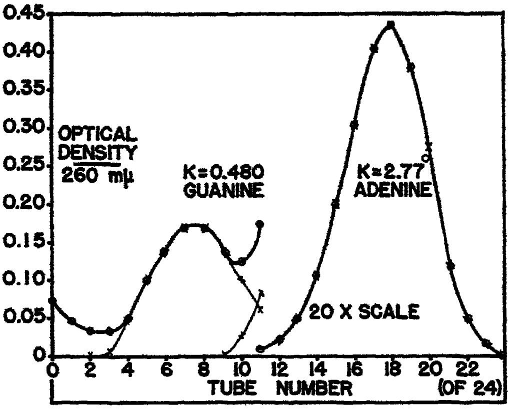

FIG. 1. Commercial adenine (initial concentration 2.65 mg. per cc.) containing about 6 per cent of guanine, twenty-four transfers. Observed points ○, calculated ×

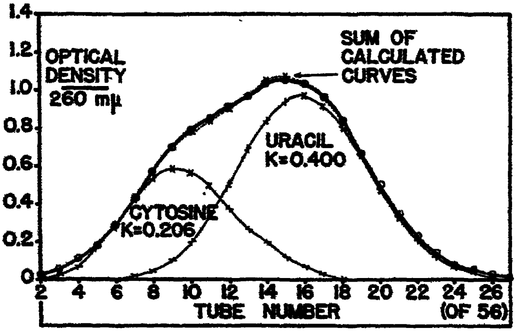

FIG. 2. Cytosine (0.20 mg. per cc.) and uracil (0.20 mg. per cc.), 56 transfers. Observed points ○, calculated ×.

DU spectrophotometer. If the solutions are more concentrated, larger volumes of ethanol may be used for the dilutions.

The optical densities at 260 mμ, or some other more appropriate wavelength, of each of the solutions were measured and were plotted against tube number, giving the distribution pattern. Calculation of the partition coefficients and construction of the theoretical curves for estimation of homogeneities were made according to Williamson and Craig (4).[1] With the exception of that of adenine, the partition coefficients have been reproducible within ±2 per cent. Complete absorption spectra were determined on selected tubes for further qualitative characterization.

The components of certain mixtures not readily separated by the solvent pair used, for instance mixtures of guanine with hypoxanthine or with uracil, have been determined by a spectrographic method. The extinction coefficients at 264.5 mμ of guanine and hypoxanthine are equal, and at 256.5 mμ those of guanine and uracil are equal. The absorption at these wave-lengths permits calculation of the total concentration of either of these pairs of substances. The values of the extinction coefficients of artificial mixtures of guanine with hypoxanthine and of guanine with uracil were determined at 285 mμ and plotted against the mole per cent of guanine. From the straight line graph thus obtained the mole per cent of guanine may be determined.

Difficulty is encountered in getting sufficient pure guanine into solution, particularly in buffers of high salt concentration, but in mixtures with other purines or pyrimidines, guanine is somewhat more soluble. The buffered solvent pair described facilitates the analysis of solutions or impure solids since pH adjustments may be readily made. For preparative distributions, it has been advantageous to use only butanol and water, in which the partition coefficients have approximately the same values, and from which reisolations may be more easily accomplished. The use of a solvent pair consisting of *n*-butanol and water with 0.1 per cent of benzyltrimethylammonium hydroxide and 28 per cent of isopropanol appears to offer certain advantages and is being further investigated.

SUMMARY

The counter-current distribution technique has been applied to purines and pyrimidines and their derivatives as a precise method of both characterization and determination of homogeneity.

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[1] We wish to express appreciation to these authors for the loan of their manuscript prior to its publication.

CRYSTALLINE ALDOLASE*

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Aldolase, the enzyme that catalyzes the reaction, fructose-1,6-diphosphate $\rightleftharpoons$ D-glyceraldehyde phosphate + dihydroxyacetone phosphate, was described and investigated in detail by Meyerhof and Lohmann (2). Herbert, Gordon, Subrahmanyan, and Green (3) purified aldolase from rabbit muscle and obtained an amorphous product which was electrophoretically homogeneous (4). Warburg and Christian (5) crystallized aldolase from rat muscle and reported a value for the "turnover number" which was about twice that found by Herbert *et al.* for their best preparation.

Seemingly unrelated to these observations was the isolation from rabbit muscle by Baranowski (6) of two crystalline proteins designated myogen A and myogen B. Baranowski assigned no enzymatic activity to these myogens and the methods he used appeared to differ from those used for the isolation of aldolase. He crystallized myogen A, in the form of hexagonal bipyramids, from ammonium sulfate solution at pH 6 and he stated that acetone could not be used successfully in the isolation of the protein. Warburg and Christian did use acetone in their fractionation procedure and they crystallized aldolase, in the form of thin six sided plates, from ammoniacal ammonium sulfate solution. Herbert *et al.* fractionated the enzyme with ammonium sulfate and stated that acetone inactivates even at 0°.

Engelhardt mentioned in a review (7) that aldolase activity had been observed in myogen A crystals. He suggested that myogen A may be identical with aldolase, but the turnover number he reported is quite low. Meyerhof and Beck (8) found that myogen A, prepared by Baranowski's procedure, showed aldolase activity that increased from about 0.1 unit per mg. of protein for the first crystals to about 0.7 unit per mg. of protein after the sixth recrystallization, but even this preparation had but one-sixth the activity reported by Warburg and Christian for crystalline rat aldolase. They concluded that myogen A is not aldolase.

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In the course of fractionation of aqueous extracts of rabbit skeletal muscle, the fraction separating between 0.50 and 0.52 saturation with ammonium sulfate at pH 7.5 was observed to consist of fine crystalline needles (Fig. 1). These could be recrystallized generally as needles, but upon occasion as large, well formed, elongated hexagonal plates (Fig. 2). Hexagonal bipyramids which resembled myogen A (Fig. 3) were obtained from rabbit muscle by a procedure which included fractionation with acetone and differed in other respects from the method outlined by Baranowski (6), except in the method of crystallization from ammonium sulfate solution at pH 5.8. Each of these three crystalline proteins proved to possess high aldolase activity.

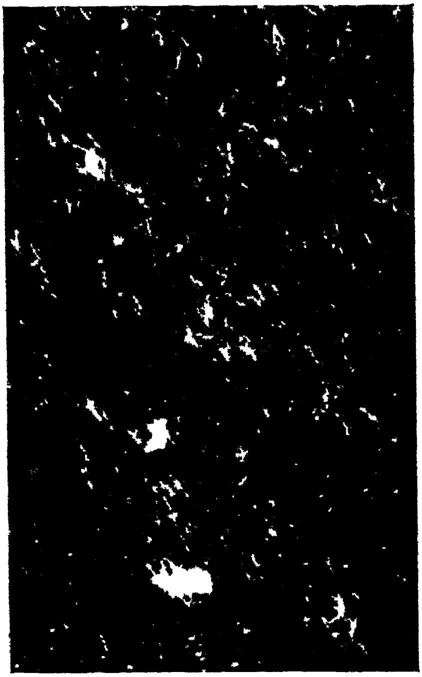

Fig. 1

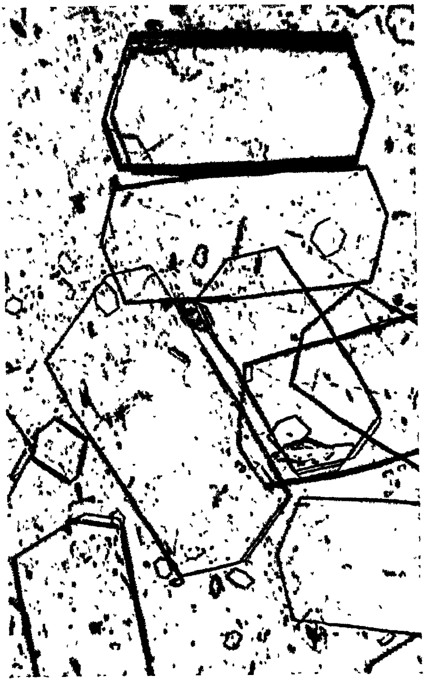

Fig. 2

Fig. 1. Crystalline rabbit aldolase, needles: dark-field; 600 ×.
Fig. 2. Crystalline rabbit aldolase; plates; 150 ×.

The bipyramids could be converted into needles and the needles, or the hexagonal plates, into bipyramids by crystallization from ammonium sulfate at pH 7.5 and 5.8 respectively. The aldolase activity of each of the three crystalline forms was the same and did not change upon interconversion.

Crystalline aldolase was also prepared from rat muscle (Fig. 4) by the method of Warburg and Christian (5). The aldolase activity of this preparation was the same as that of the rabbit protein when either of two test systems was used, one based on chemical analysis, the other on optical measurements.

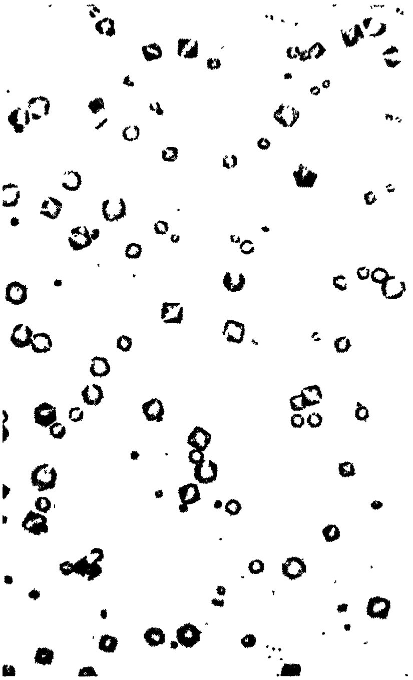

FIG. 3

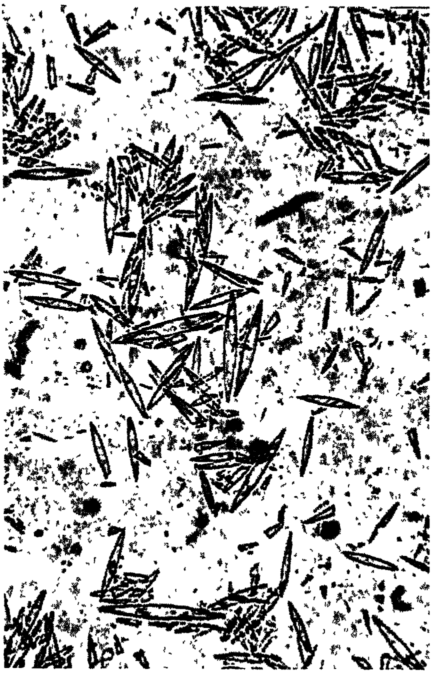

FIG. 4

FIG. 3. Crystalline rabbit aldolase, hexagonal bipyramids prepared from needles; 150 ×.

FIG. 4. Crystalline rat aldolase; 150 ×.

EXPERIMENTAL

Aldolase from Rabbit Skeletal Muscle; Crystallization As Needles—This method, because of its simplicity and reproducibility, is recommended for the isolation of aldolase. Over 60 preparations according to this method have been carried out in this laboratory. The ground skeletal muscle of a rabbit is extracted in a cold room at 5° with two equal portions of cold water (or cold 0.03 N NaOH or KOH) and strained through gauze. The cold extract is first brought to pH 7.5 with dilute NaOH and then to 0.5 saturation by the addition of an equal volume of $(NH_4)_2SO_4$ solution (saturated at room temperature and adjusted to pH 7.5 by the addition of concentrated NH_3). The solution is cooled to 0° and the precipitate is removed by filtration in the cold room. To the clear filtrate is added enough saturated $(NH_4)_2SO_4$ solution, pH 7.5, to make the saturation 0.52 (4 ml. of $(NH_4)_2SO_4$ solution for each 100 ml. of filtrate). The solution is stirred frequently while standing in the cold. After some hours, often 12 or more, the presence of fine crystals can be detected by the "sheen" when the solution is stirred; the crystals do not settle on standing. Initial crystallization may be speeded by allowing the solution to warm to room temperature, followed by a return to the cold room. Further standing for several days is advisable for a maximum yield. The crystals can then be removed by filtration or high speed centrifugation. The sample protocol in Table I shows that the separation is sharp, and that the activity of the first crystals is high and increases but slightly on recrystallization. The yield of first crystals corresponds to about 75 per cent of the aldolase activity in the crude aqueous extract and amounts to 10 per cent of the extracted proteins or 0.35 gm. per 100 gm. of muscle.

TABLE I

Crystallization of Aldolase; Sample Protocol

		Protein	Enzyme activity	Units per mg. protein
		gm.	*units*	
Extract from 500 gm. of rabbit skeletal muscle		24.0	2136	0.089
	Saturation			
Fractionation with $(NH_4)_2SO_4$	0 -0.40	4.1	131	0.031
	0.40-0.50	0.6	91	0.152
	0.50-0.52*	2.43	1631	0.671
	Supernatant fluid	17.1	205	0.012
Total recovered........		24.2	2058	
0.50-0.52 fraction, recrystallized..				0.684

* Crystalline.

Recrystallization—The precipitate of fine needles is dissolved in a little water and brought to pH 7.5. A small amount of amorphous material may be removed by bringing the solution to incipient turbidity with $(NH_4)_2SO_4$ at pH 7.5 and letting it stand for several hours in the cold. Crystals begin to separate upon the cautious addition of saturated $(NH_4)_2SO_4$ solution, pH 7.5, at less than 0.5 saturation in the cold. The protein can be recrystallized at room temperature in the same way. Since this protein is less soluble at room temperature than in the cold, at concentrations of $(NH_4)_2SO_4$ higher than about 0.4 saturation, it can also be recrystallized with the aid of this property.

These fine crystals are difficult to see under the microscope, since the contrast is slight. It has been impossible to make certain that their appearance as needles is not misleading, and that they are not, in reality, extremely thin plates seen on edge. Their appearance in a dark field, of which Fig. 1 is an inadequate representation, seems to indicate needles.

Crystallization As Plates—When aldolase needles are dissolved in water, brought in the cold to incipient turbidity with $(NH_4)_2SO_4$ at pH 7.5 (about 0.4 saturation), and allowed to warm gradually to room temperature, the protein has occasionally been found to separate as beautiful large six sided plates (Fig. 2). The plate form has been recovered upon recrystallization, with seeding, but it has not proved possible to convert the needle form to the plates every time it has been attempted. The factors which control this behavior have not been determined. The plates as well as the needles can be converted to bipyramids (see below).

Crystallization As Bipyramids—These crystals were obtained from rabbit muscle as a by-product in the purification of the enzyme phosphoglucomutase. An extract was prepared as described above and all steps of the fractionation were carried out in a cold room at 5°. The precipitate that formed between 0.4 and 0.6 saturation with $(NH_4)_2SO_4$ at pH 7 was collected by filtration and dissolved in 1 per cent sodium glycerophosphate solution (5 volumes of solution to 1 volume of precipitate). To this solution, cooled to 0°, ice-cold acetone was slowly added to a concentration of 41 per cent, the precipitate was discarded by centrifugation, and the concentration of acetone in the supernatant fluid brought to 55 per cent. After the second acetone precipitate was dissolved in 1 per cent glycerophosphate solution, the successive fractionation with $(NH_4)_2SO_4$ (between 0.4 and 0.6 saturation) and with acetone (between 40 and 55 per cent) was repeated. The acetone precipitate was dissolved in 0.3 saturated $(NH_4)_2SO_4$, pH 7, insoluble material was discarded, and the supernatant fluid was brought to 0.45 saturation. It was at this step in the procedure that aldolase could be separated from phosphoglucomutase. The cold 0.45 saturated $(NH_4)_2SO_4$ solution of protein was warmed to 25°, with constant stirring, by being placed in a water bath at 30°. The heavy precipitate that formed (which could be redissolved completely by cooling the solution again to 0°) was separated by centrifugation at room temperature and dissolved in a small volume of ice-cold water. To the clear solution, saturated $(NH_4)_2SO_4$ solution (pH about 5.7) was added dropwise with stirring until a faint turbidity had developed. It was then left at 5°. Bipyramidal six sided crystals appeared after several days. Further addition of saturated $(NH_4)_2SO_4$ solution led to further crystallization. These crystals were very large and were free of visible amorphous "background" upon microscopic observation.[1] The crystals were centrifuged in the cold and brought into solution in ice-cold water in which they dissolve very slowly. Recrystallization from a solution containing 1 per cent protein, or more, was rapid when saturated $(NH_4)_2SO_4$ solution was added to faint turbidity and the solution was seeded. The second crystals were smaller than the first.

The hexagonal bipyramids shown in Fig. 3 were actually prepared from aldolase needles, but cannot be distinguished by ordinary visual observation from the recrystallized bipyramids prepared as described above. The several varieties of hexagonal bipyramids, described by Chrobak and Baranowski (9) for their preparation of myogen A, have not been detected among our crystals.

Interconversion—To a clear solution, prepared from the aldolase needles and containing about 2 per cent protein, saturated $(NH_4)_2SO_4$ solution that has not been neutralized (about pH 5.7) is added in the cold until a very faint turbidity persists. This is ordinarily sufficient to bring the entire solution to pH 6 or slightly less. Typical hexagonal bipyramids separate in 20 to 48 hours if the solution is seeded (Fig. 3). These crystals, or the bipyramids prepared directly from the muscle, dissolved in water, can easily be converted to needles by crystallization from $(NH_4)_2SO_4$ at pH 7.5.

Crystalline Aldolase from Rat Skeletal Muscle—The skeletal muscle from nine rats, amounting to 780 gm., was ground and extracted with water. All subsequent operations were performed at 0–4° and followed the procedure outlined by Warburg and Christian (5), except as mentioned. After the preliminary fractionation with acetone had been completed, it was found that the protein was more soluble in the indicated concentrations of $(NH_4)_2SO_4$ at 0–4° than was anticipated from Warburg and Christian's directions, while upon raising the temperature of the solution to about 20° much of the protein precipitated. This fraction was separated by centrifugation at room temperature and preserved, since the precipitation on warming resembled the behavior of rabbit aldolase. Fractionation of the rest of the material according to Warburg and Christian (5) was continued at low temperature. After two fractionations with ammoniacal $(NH_4)_2SO_4$, instead of the four which they described, it proved possible to crystallize both the fraction separated by warming and the one fractionated at low temperature. The crystallization was accomplished with ease by adding saturated ammoniacal $(NH_4)_2SO_4$ solution to the ice-cold concentrated protein solution until faint turbidity developed and then allowing the solution to warm slowly to room temperature. Recrystallization followed the same procedure. The crystals (Fig. 4) have the same characteristic appearance as those shown in the paper by Warburg and Christian (5).

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[1] In our experience the first myogen A crystals obtained by the Baranowski procedure (6) are usually contaminated with a considerable amount of amorphous material, which continues to appear on successive recrystallizations.

We did not investigate in this preparation whether any of the other steps in the Warburg and Christian procedure might be eliminated, except that the protein separated by warming crystallized without having been treated with alumina cream; these crystals had the same aldolase activity as those prepared by the more elaborate method, including alumina cream treatment.

Aldolase Activity; Chemical Test—The chemical test used by Herbert *et al.* (3) has been modified for convenience in several particulars. The substrate solution contains 0.01 M fructose-1,6-diphosphate[2] and 0.1 M glycine buffer at pH 9.0. The enzyme solution is prepared by dilution with 0.1 M KCN solution acidified to pH 9.0.[3] To 0.1 ml. of substrate solution in a Pyrex test-tube in a bath at 30° is added 0.1 ml. of enzyme solution and the reaction is allowed to proceed for 5 minutes. It is stopped by the addition of 1.0 ml. of 1 N NaOH, freshly prepared each day (to avoid SiO_2). After 15 minutes at room temperature, for the hydrolysis of the alkali-labile triose phosphate formed in the reaction, the solution is neutralized with an equivalent amount of H_2SO_4. The reagents for the determination of inorganic phosphate according to Fiske and Subbarow (11) are then added directly, the final volume being 10 ml. The color is measured in the Klett photocolorimeter with Filter 660.

Optical Test—The crystalline D-glyceraldehyde phosphate dehydrogenase from rabbit muscle (12) has been used, instead of the corresponding yeast enzyme, in the optical test described by Warburg and Christian (5). Since the dehydrogenase is present in excess, the aldolase concentration determines the rate of the reaction. The rate of reduction of diphosphopyridine nucleotide (DPN) is followed by measuring the absorption of light at 340 mμ with the Beckman spectrophotometer. The concentrations of reagents are those used by Warburg and Christian, except that of DPN, which is lower. Tests showed that when DPN was added in one-half the usual concentration this did not affect the rate. The concentration of DPN has been determined at 340 mμ after complete enzymatic reduction by glyceraldehyde phosphate in the presence of the dehydrogenase and arsenate. Ohlmeyer's value of the extinction coefficient (13) was used in the calculations.

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[2] Fructose-1,6-diphosphate (a commercial sample of the calcium salt obtained from the Schwarz Laboratories) gave a high blank in the chemical test. It was purified by precipitation as the monobarium salt (10) and converted to a solution of the sodium salt for use.

[3] In order to extend the range of proportionality of enzyme action on dilution, it is desirable to add a protective protein. 0.2 mg. of recrystallized human serum albumin in 1 ml. of cyanide solution has been found satisfactory.

The value of the aldolase activity at a fixed temperature is not easily obtained in the optical test, since the temperature rises considerably within the absorption cell of the Beckman spectrophotometer as the latter is constructed at present. The temperature has been measured within the cell at the end of each experiment and the data have been corrected to 30° with the aid of the temperature coefficients given by Herbert *et al.* (3). Concordant values upon duplicate experiments are thus obtained.

Protein concentrations in solutions of crystalline aldolase were determined optically, from the absorption of light at 280 mμ measured in the Beckman spectrophotometer. Protein concentration (mg. per ml.) = $(-\log_{10} T)/Kl$ where $K = 0.806$ and l is in cm. Protein concentrations, especially in crude fractions, were also determined by the quantitative biuret method of Robinson and Hogden (14).

The results have been expressed in Table II both in aldolase units and as a turnover number. 1 unit represents 1 mg. of P transformed in 1 minute, under the experimental conditions shown in Table II. The turnover number has been calculated in terms of the number of moles of hexose diphosphate transformed in 1 minute by 150,000 gm. of protein.[4] Since each of the previous workers had used somewhat different methods of expressing activity, the results from the literature given in Table II have been recalculated in the terms just described.

No details are given by Engelhardt (7) as to how the value for the turnover number of aldolase was obtained, but in any case the value is very low. The turnover number given for myogen A by Meyerhof and Beck (8) at 37° is the same as that we have found at 30°. Since the temperature coefficient, Q_{10}, is close to 2 (3), it follows that the protein described in this paper is considerably more active than repeatedly recrystallized myogen A.

According to their chemical test, the preparations of Herbert *et al.* (3) appear to be more active at 30° than our preparations, but this is probably due to differences in the methods used for measuring activity rather than in the purity of the preparations. That the chemical test may need reexamination is indicated by the fact that we have obtained at pH 9 in the chemical test the same activity as at pH 7.6 in the optical test. Since aldolase is about 25 per cent more active at pH 9 than at pH 7.6 (3), it would appear that our chemical test gives values which are too low.

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[4] This figure was chosen to represent the molecular weight on the basis of Gralén's results with myogen A (15). The minimum molecular weight calculated from amino acid analyses of aldolase (16) is of the same order of magnitude and the diffusion constants of the two proteins agree closely.

Warburg and Christian (5) have pointed out that their rat aldolase preparations were twice as active as the rabbit aldolase preparations of Herbert *et al.* (3). This might have been due to species difference, but the data in Table II show that our rat and rabbit preparations have the same activity, both in the chemical and in the optical tests.

With the same optical method as that used by Warburg and Christian we found at 30° about 40 per cent of the activity, for crystalline rat aldolase, that might have been expected from the measurements of these authors at 20° and 38°. We have explored the effect of variations in concentrations of substrate and other components of the reaction mixture without detecting significant differences. In particular, the presence of cysteine is necessary for the full activity of the glyceraldehyde phosphate dehydrogenase from muscle (12), which we used in the optical test. Warburg and Christian (5), who used the corresponding enzyme from yeast, state that in their optical test cysteine was ordinarily used, except when the aldolase had been crystallized. To avoid the possibility that the known reaction of cysteine with glyceraldehyde phosphate might have interfered, we have substituted glutathione with identical results.

TABLE II

Aldolase Activities of Various Preparations

Investigators	Source	*t*	pH	Chemical test	Optical test	Turnover No.
		°*C.*		*units per mg. protein*	*units per mg. protein*	
Engelhardt (7)						1,000*
Meyerhof and Beck (8)	Rabbit	37	†	0.7		1,690
Herbert *et al.* (3)	"	30	7.3	1.1‡		2,680‡
		38	7.3	2.0		4,950
Warburg and Christian (5)	Rat	20	7.6		0.89	2,150
		38	7.6		4.31	10,430
This investigation	Rabbit	30	7.6		0.70	1,690
		30	9.0	0.69		1,670
	Rat	30	7.6		0.73	1,770
		30	9.0	0.69		1,670

* Not recalculated. Details of the original measurement and calculation not given (7).

† pH not clearly stated but presumably near 7.6.

‡ Estimated from Fig. 3, Herbert *et al.* (3).

The reason for the discrepancy between the values of Warburg and Christian and our own has not been explained. The data given in the next section show that the proteins from both rat and rabbit muscle proved to be electrophoretically homogeneous over a wide pH range.

In view of the fact that the same activity was obtained for each of the three crystalline modifications of the rabbit protein, that the activity remained the same on interconversion of these different crystal forms, and that the same activity was also obtained for rat aldolase crystallized according to Warburg and Christian, we are of the opinion that these proteins represent the enzyme aldolase. Further work is in progress to examine the exact relation between the myogen A preparations of Baranowski and our preparation of aldolase.

Electrophoresis—The electrophoretic mobility and homogeneity of crystalline preparations of aldolase have been investigated in the Tiselius apparatus (17) equipped with the long center section (Longsworth *et al.* (18)). The cylindrical lens optical system (Philpot (19)) has ordinarily been used. Experiments have been performed at 2°, over the pH range 5.2 to 8.6, in acetate, phosphate, or veronal buffers of ionic strength, $\Gamma/2$, 0.1. Phosphate buffers were prepared according to Green (20); all were checked with a glass electrode pH meter, sensitive to ±0.02 pH. The pH of 0.05 M potassium acid phthalate was taken as 4.00 (at about 20°).

From a comparison of the electrophoretic schlieren diagrams for the rabbit muscle extract and for aldolase (Fig. 5, *a* and *b*), it may be noted that aldolase forms part of the large protein component that moves most slowly at pH 7.4. D-Glyceraldehyde-3-phosphate dehydrogenase also forms part of this component (12).

Fig. 5, *b*, obtained with first crystals from rabbit, shows a main component and a small, fast moving peak, which is absent after recrystallization, while the main peak remains unchanged. On the descending schlieren diagram of Fig. 5, *b*, there will be noted a thin "spike" on the side of the main peak. This has appeared in every electrophoretic diagram of recrystallized rabbit or rat aldolase on the descending side only.

The appearance of the spike is similar to that of the so called "β-anomaly" observed in electrophoretic diagrams obtained with blood plasma (21). In a similar fashion the spike appears to be associated with the appearance of a thin plane of turbidity which can just be detected at the descending boundary by direct inspection of the cell. The appearance of the spike during electrophoresis may mean that aldolase itself precipitates under the influence of the conditions that obtain in the descending boundary. There is no evidence at present to implicate a lipide or lipoprotein; extraction of aldolase with ether in the cold is without effect on the spike, while the analytical data on the dried protein leave little room for such a component (16).

With the exception of the spike phenomenon, the recrystallized rabbit protein showed a high degree of electrophoretic homogeneity over the pH range 5.2 to 8.6.

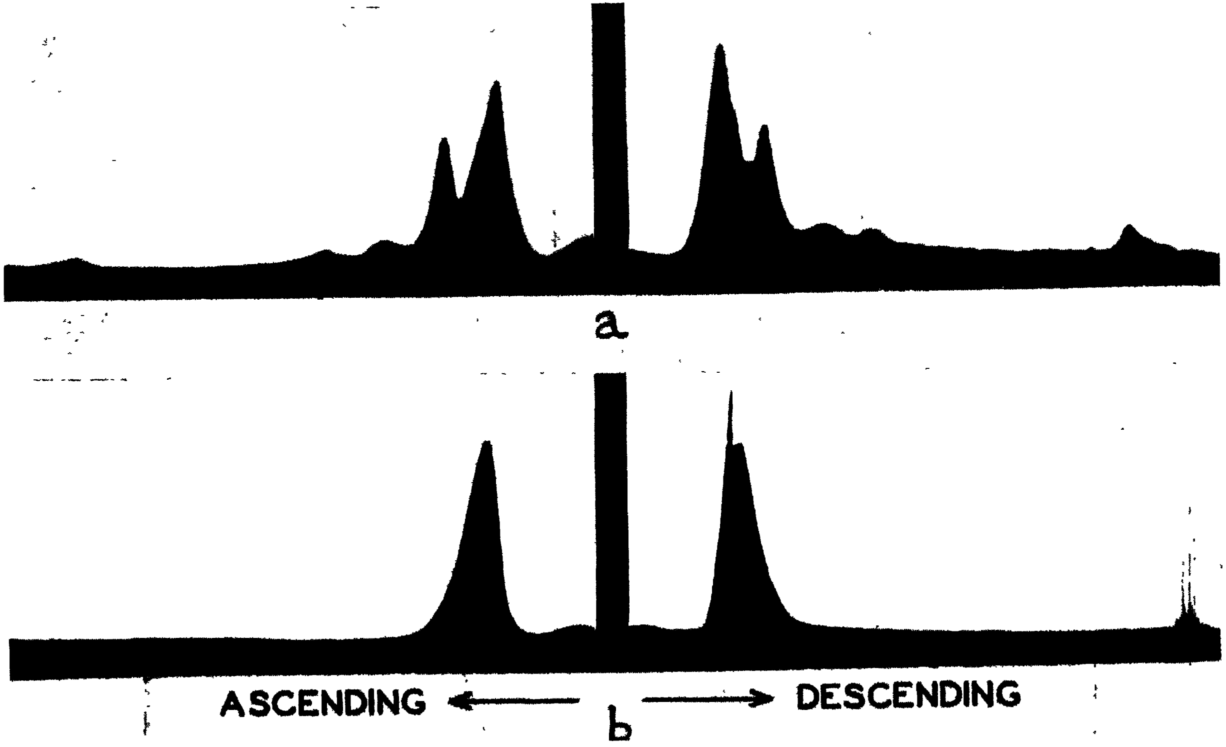

FIG. 5. Electrophoretic schlieren diagrams in phosphate buffer, pH 7.4, ionic strength 0.1, after 4 hours at 2°. *a*, original extract of muscle; *b*, fraction crystallized between 0.50 and 0.52 saturation with $(NH_4)_2SO_4$ at pH 7.5.

Fig. 6 represents the variation in the electrophoretic mobility of rabbit aldolase from pH 5.7 to 7.8. The ascending and descending mobilities (corrected for cylindrical lens error) are generally quite close, and have been averaged. In several instances rabbit aldolase crystallized both as needles and as bipyramids proved to have the same mobility under identical conditions. The points have been connected by a smooth free-hand curve, establishing the isoelectric point under these conditions at pH 6.05. Bate-Smith (4) determined the electrophoretic mobility of a purified aldolase prepared by Herbert *et al.* (3) at pH 6.0 and 7.0 and estimated the isoelectric point to be about pH 6.3.

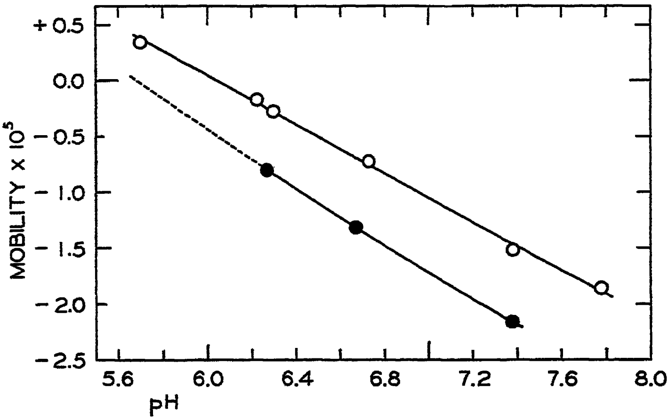

FIG. 6. Electrophoretic mobility of crystalline aldolase in sq. cm. volt^{-1} sec.$^{-1}$ × 10^5 at 2° in phosphate buffers of ionic strength 0.1; ○ rabbit, ● rat.

The mobility of rat aldolase is considerably greater than that of rabbit aldolase at three pH values and under the same conditions. Extrapolation, as indicated in Fig. 6, would give the isoelectric point of the rat protein as pH 5.7. The rat aldolase is also electrophoretically homogeneous over this pH range.

Mobilities observed in acetate buffer, pH 5.2 and 5.6, and in diethyl barbiturate buffer, pH 8.6, do not fall on the curve for phosphate buffer shown in Fig. 6. Specific effects of buffer species on mobility have been reported in other instances (*e.g.*, Davis and Cohn (22)).

Diffusion—The diffusion constant of rabbit aldolase has been measured in the electrophoresis cell with long center section as described by Longsworth (23) and tested in this laboratory by Green (24). Phosphate buffer, $\Gamma/2 = 0.1$, pH $= 7.1$, was used and the experiments were performed at 2°. Two separate experiments were carried out. The results were computed separately for each of the two limbs of the cell, from enlarged tracings of schlieren scanning photographs, according to the relations $D_A = A^2/4\pi t H_m^2$ and $D_\mu = \mu^2/2t$, where A is the area under the diffusion diagram in sq. cm., t is the time in seconds, H_m is the maximum height of the curve in cm., and μ is one-half the breadth of the curve, in cm., at its inflection point, where $H_\mu = H_m/\sqrt{e}$. The values obtained at a number of different times were essentially constant during an experiment lasting 220 hours. The method of moments, applied to a few individual diagrams, gave values consistent with those obtained by the other two methods. A few curves were also compared with normal distribution curves, with satisfactory agreement (25).

The average value in water at 20° was for D_A 4.58 and for D_μ 4.68 × 10^{-7} sq. cm. sec.$^{-1}$. The average, 4.63, is quite close to the value of $D_{20,w} = 4.78 \times 10^{-7}$ sq. cm. sec.$^{-1}$ obtained by Gralén in a study of the physical constants of crystalline myogen A from rabbit muscle (15).

The authors wish to thank Mr. Robert Loeffel for assistance in carrying out a number of the diffusion and electrophoresis measurements reported in this paper.

SUMMARY

1. The enzyme aldolase has been isolated from rabbit skeletal muscle in the form of fine needles or hexagonal plates by crystallization from $(NH_4)_2SO_4$ at pH 7.5 or in the form of hexagonal bipyramids by crystallization from $(NH_4)_2SO_4$ at pH 5.8. These crystal forms can be interconverted by crystallization at the appropriate pH without change in the specific aldolase activity. The turnover number corresponds to the splitting of 1670 moles of fructose diphosphate by 150,000 gm. of protein in 1 minute at 30° and pH 7.6. Aldolase crystallized from rat muscle has the same turnover number.

2. About 75 per cent of the aldolase activity and about 10 per cent of the protein in an aqueous extract of rabbit muscle can be recovered as aldolase crystals by fractionation between 0.50 and 0.52 saturation with $(NH_4)_2SO_4$ at pH 7.5.

3. Both rabbit and rat aldolase are electrophoretically homogeneous over a wide pH range. The isoelectric points in phosphate buffer of ionic strength 0.1 are 6.05 and 5.7, respectively.

4. The diffusion constant of crystalline rabbit aldolase is 4.63 × 10^{-7} sq. cm. sec.$^{-1}$ in water at 20°.

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CRYSTALLINE D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM RABBIT MUSCLE*

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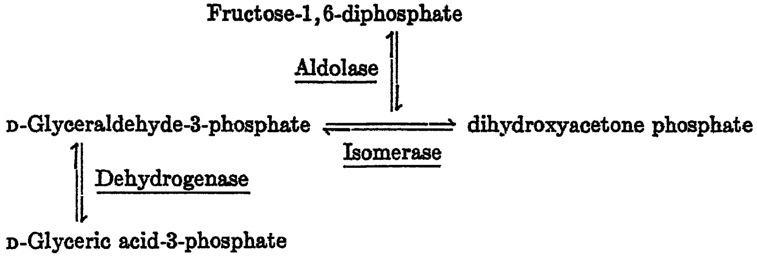
The reaction catalyzed by this enzyme consists in the oxidation of a —CHO to a —COO·PO_3H_2 group and represents the first oxidative step in the degradation of carbohydrate in the tissues. Diphosphopyridine nucleotide (DPN) and inorganic phosphate are necessary for this reversible enzymatic reaction. The equilibrium has been investigated by Warburg and Christian (1), Drabkin and Meyerhof (2), and Meyerhof and Oesper (3). When inorganic phosphate is replaced by arsenate, the reaction becomes irreversible. In both instances the reaction can be followed by measuring the appearance of reduced DPN spectrophotometrically at 340 mμ, a method originally introduced by Warburg and Christian.

The substrate for this enzyme is formed from fructose-1,6-diphosphate through the action of aldolase, the enzyme described in the preceding paper.


Fructose-1,6-diphosphate
Aldolase
D-Glyceraldehyde-3-phosphate
dihydroxyacetone phosphate
Isomerase
Dehydrogenase
D-Glyceric acid-3-phosphate

This sequence of reactions, originally proposed by Embden and Meyerhof, could be demonstrated by means of the crystalline enzyme preparations because they are free of isomerase (Fig. 1). In the presence of aldolase, dehydrogenase, arsenate, and DPN, glyceraldehyde phosphate only disappears and the yield is 1 mole of triose phosphate oxidized per mole of hexose diphosphate added; when, in addition, a purified preparation of triose phosphate isomerase (5) is added, dihydroxyacetone phosphate disappears and a second mole of triose phosphate is oxidized.[1]

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[1] This provides a sensitive method for the quantitative determination of hexose diphosphate and triose phosphate, either separately or combined, depending on the order of addition of the three enzymes. This method will be described in a subsequent publication.

The preparation of the crystalline enzyme was described in a preliminary report (6) and shortly thereafter a note appeared by Dixon and Caputto (7) in which crystallization was achieved by another method of preparation. The enzyme had previously been crystallized from yeast by Warburg and Christian (1). In confirmation of them it was found that the enzyme, when used in much higher concentration, also oxidizes D-glyceraldehyde.

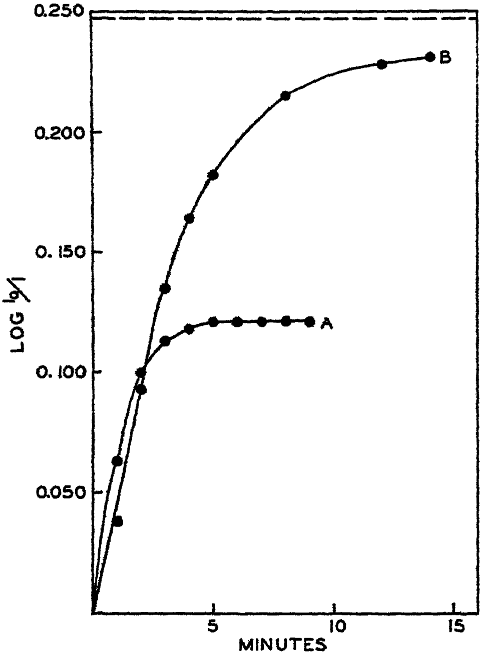

FIG. 1. Spectrophotometric measurement of triose phosphate oxidation with fructose diphosphate as substrate. Curve A, the reaction mixture consisted of fructose diphosphate, aldolase, dehydrogenase, DPN, and arsenate. 1 mole of triose phosphate was oxidized (or DPN reduced) per mole of fructose diphosphate added. Curve B, the reaction mixture contained in addition triose phosphate isomerase. 2 moles of triose phosphate were oxidized per mole of fructose diphosphate. The dotted line indicates the amount of reduction of DPN expected from the amount of fructose diphosphate added. Fructose diphosphate was determined by the phenylhydrazine method of Deuticke and Hollmann (4).

The present paper contains details of preparation, data on the properties of the enzyme protein, and kinetic measurements, while the papers which follow contain data on the prosthetic group and on amino acid composition. The enzyme constitutes about 7 to 12 per cent of the extracted proteins and the yield is about 300 mg. per 100 gm. of muscle.

EXPERIMENTAL

Method of Preparation—A rabbit is injected intravenously with a lethal dose of amytal. It is rapidly skinned and the leg and back muscles are excised and weighed. All further steps are carried out in a cold room. The muscles are passed through a meat grinder, extracted immediately with 1 volume of 0.03 N KOH for 10 minutes with occasional stirring, and strained through gauze. The extraction is repeated and the residue is suspended in 0.5 volume of water for 5 minutes and strained as above. The pH, measured with the glass electrode in the combined extracts, varied in six different preparations from 6.6 to 7.2.

If it is desired to prepare both aldolase and dehydrogenase, 1 volume of saturated $(NH_4)_2SO_4$ solution, pH 7.5 to 7.8 (saturated at room temperature and pH adjusted with ammonia), is added to the extract (0.5 saturation) and the mixture placed in an ice bath for about 0.5 hour. From the time of killing the rabbit to the addition of the salt solution not more than 1.5 hours should elapse. The mixture is filtered through folded paper (Whatman No. 1), and to the clear filtrate more saturated $(NH_4)_2SO_4$ solution is added to bring the saturation to 0.52 (4 ml. to each 100 ml.). The pH should be 7.6 to 7.8. This solution either is left at 5° for 2 to 3 days or is warmed up very slowly to 20°, then returned to 5°. This procedure speeds up the rate of crystallization of aldolase (8). When a heavy mass of aldolase crystals (which do not settle) has formed, they are separated by filtration or centrifugation.

When it is not desired to prepare aldolase, saturated $(NH_4)_2SO_4$ solution is added to 0.52 saturation in one step, followed immediately by filtration or by centrifugation in a high speed angle centrifuge.

To each 100 ml. of filtrate at 0.52 saturation are added 13 gm. of solid $(NH_4)_2SO_4$, which brings the saturation to 0.72. As soon as the salt is dissolved by gentle stirring, the solution is filtered through folded paper, the filtrate being poured back on the filter until it is perfectly clear. Filtration is fairly rapid and the pH of the filtrate is about 7.5. To the filtrate, 15 per cent ammonium hydroxide solution is added dropwise with shaking until the pH is 8.2 to 8.4. The pH is measured with a glass electrode, or with metacresol purple as indicator, in an aliquot of the 5 times diluted filtrate. Crystals appear in several hours, and even without seeding a large crop of crystals (which do not settle) forms overnight. The suspension of the crystals may be left standing for several days to increase the yield. The crystals are separated by filtration through folded paper (Whatman No. 1). Filtration is rather rapid at first but eventually slows down; by the use of an automatic filtration device all the material from 300 to 500 gm. of muscle will pass through a filter of 24 cm. diameter overnight. The still moist crystalline precipitate is scraped off the paper with a spatula and dissolved in 40 to 80 ml. of water. Paper fibers, $MgNH_4PO_4$ crystals, and shreds are removed by centrifugation. To the clear, slightly reddish yellow solution are added 2 volumes of saturated $(NH_4)_2SO_4$ solution, pH 8.2 to 8.4, for each volume of water used in the solution of the crystals. There is no immediate precipitation of protein. In less than 1 hour, crystals begin to appear and in 24 hours a thick shimmering suspension of crystals has formed. Further recrystallizations are carried out in the same manner. The yellowish color is eventually removed with the mother liquors and the suspension appears pure white. By this procedure thirty-four preparations of the enzyme have been made in the past 2 years. There was no case of failure and the yield was 1 gm. or more of enzyme per rabbit.[2]

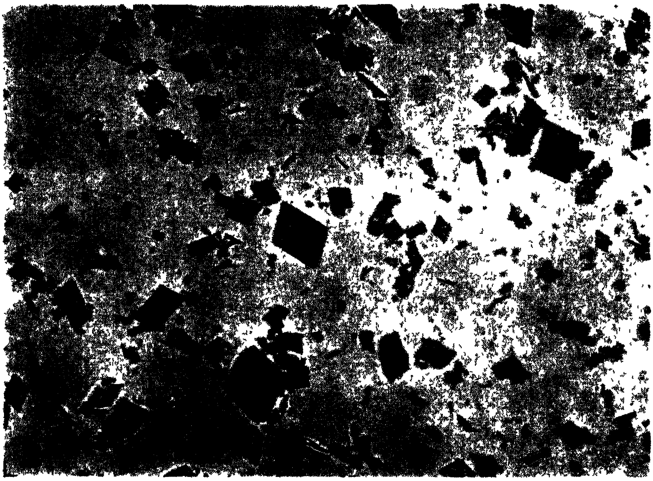

FIG. 2. Crystalline D-glyceraldehyde-3-phosphate dehydrogenase; × 260

The first crystals usually appear in the form of rosettes, which are apparently made up of clusters of fine needles. Closer observation reveals that the crystals consist of diamond-shaped plates which stand on edge. On recrystallization the crystals remain separate. Because of the thinness of the plates and the closeness of the refractive indices of the crystals and the mother liquor, satisfactory photomicrographs were obtained only when the crystals were stained with methylene blue. The crystals shown in Fig.

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[2] It should be emphasized that, in order to obtain a good yield of crystalline dehydrogenase, the rapidly excised muscles must be ground and extracted without delay. When the muscles were left at 5° for 1 hour, or when 0.5 hour elapsed between the time the muscles were ground and placed in the extraction fluid, no, or only a small, yield of crystalline enzyme could be obtained.

2 were obtained by allowing the enzyme to recrystallize slowly from 0.6 saturated $(NH_4)_2SO_4$ solution and are larger than those ordinarily obtained from 0.66 saturated $(NH_4)_2SO_4$. Both the diamond shape of the crystals and the simulated rod shape due to the crystals standing on edge are seen in the photograph.

An example of the yield and activity on recrystallization is given in Table I. It may be seen that the enzyme crystallizes from a 0.14 per cent protein solution in which it constitutes about one-half of the protein present. About 10 per cent of the enzyme is lost in the mother liquor, while on recrystallization from more concentrated solutions the loss is 5 to 8 per cent. The specific activity of the crystals increases slightly on recrystallization, while that of the mother liquor increases very markedly and approaches that of the crystals. The enzyme is not completely stable when in solution in ammonium sulfate and this may account for the fact that the specific activity in the mother liquor did not reach that of the crystals.

TABLE I

Recrystallization of Dehydrogenase

The enzyme was prepared from 500 gm. of rabbit muscle by the method described in the text.

Crystallization	Protein in crystals	Mother liquor		Specific activity*	
		Volume	Protein content	Crystals	Mother liquor
	gm.	*ml.*	*gm.*		
1st.	1.56	2100	1.42	9.15×10^8	0.94×10^8
2nd.	1.43	206	0.13	8.64×10^8	2.92×10^8
3rd.	1.35	214	0.08	10.1×10^8	6.66×10^8

* The specific activity (at 25°) was obtained by dividing the bimolecular rate constants by the mg. of protein per ml. of reaction mixture.

Why 0.03 N alkali rather than water is used for the extraction of muscle in the preparation of the dehydrogenase is shown in the following experiment.

1 part of ground rabbit muscle was extracted with water (pH of extract 6.1) and another part with 0.03 N KOH (pH of extract 7.1), followed in each case by the procedure outlined for the preparation of the dehydrogenase. The water extract yielded only a trace of crystals, while a large crop was obtained from the alkaline extract. Both preparations were analyzed for protein and specific activity by sampling the well mixed 0.72 saturated $(NH_4)_2SO_4$ solution. The water extract yielded 0.69 mg. of protein per ml. and a specific activity of 5.12×10^8; the respective values for the alkaline extract were 1.43 mg. of protein and 8.55×10^8 specific

activity. From this it may be calculated that the extract prepared with alkali contained 3.5 times more enzyme than the water extract, and it is therefore not surprising that few crystals were obtained from the latter. Further investigation is required to explain the low yield of the enzyme from water extracts.

Electrophoresis—The electrophoretic behavior of D-glyceraldehyde phosphate dehydrogenase has been investigated with the technique described for aldolase (8). Experiments have been performed at 2° over the pH range 5.1 to 8.45.

The recrystallized enzyme is electrophoretically homogeneous over the pH range 6.2 to 7.7. Fig. 3, from an experiment at pH 7.4, shows a single peak exhibiting a slight skew. It was found that the enzyme solution (7 mg. per ml.) had lost 7 per cent of its activity during the dialysis period of 20 hours which preceded the electrophoresis. In solutions more acid than pH 6.2 or more alkaline than pH 7.7, instability of the enzyme interferes with electrophoretic measurements. When the enzyme was dialyzed against acetate buffers of pH 5.1 to 5.4 for 20 hours, it lost about 70 per cent of its activity. No appreciable turbidity developed, but a considerable part of the protein became insoluble in 0.3 saturated $(NH_4)_2SO_4$ solution at pH 8.2, indicating denaturation. During electrophoresis at this low pH range several minor peaks appeared besides a major one. In veronal buffer of pH 8.45, pronounced turbidity developed during electrophoresis, nearly masking the moving boundaries.

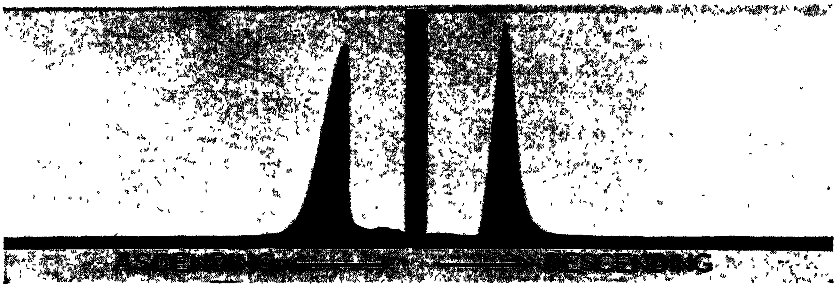

FIG. 3. Electrophoretic schlieren diagram of recrystallized D-glyceraldehyde-3-phosphate dehydrogenase after 4 hours at 2° in phosphate buffer, pH 7.4, ionic strength 0.1.

Fig. 4 shows the variation in electrophoretic mobility from pH 6.2 to 7.7 in phosphate buffer of ionic strength 0.1. The ascending and descending mobilities were generally quite close, and have been averaged. A free-hand curve drawn through the points establishes the isoelectric point under these conditions at pH 6.55.

Activity Measurements—A portion of the crystal suspension is centrifuged sharply, drained, and dissolved in 0.03 M sodium pyrophosphate, pH 8.5, in the cold to give a concentration not less than 1 mg. of protein per ml. This stock solution is stable for 2 to 3 hours at 0°, but is unstable at room temperature. For activity tests this solution is diluted with pyrophosphate buffer containing cysteine. Dilution in the absence of cysteine results in loss of enzyme activity. Cysteine cannot be replaced by a protective protein such as crystalline serum albumin. To give an example (which incidentally describes the procedure usually followed), 0.1 ml. of a stock solution (410 γ of protein) was diluted to 4 ml. (*a*) with 0.03 M pyrophosphate, pH 8.5, (*b*) with 0.004 M cysteine in 0.03 M pyrophosphate, (*c*) with 0.03 M pyrophosphate containing 225 γ of serum albumin, and (*d*) with a solution containing cysteine, pyrophosphate, and albumin. As soon as each dilution was made, 0.1 ml. (10 γ) was transferred to a silica absorption cell containing 2.7 ml. of a 0.004 M cysteine-0.03 M pyrophosphate-DPN mixture. After the mixture stood for 7 minutes at room temperature, the reaction was started by the addition of 0.2 ml. of a mixture of triose phosphate and arsenate. Density readings at 340 mμ were taken at 1 minute intervals in the Beckman spectrophotometer. Bimolecular rate constants were calculated from the 1 and 2 minute readings. These showed that Sample *a* had 45 and Sample *c* 23 per cent less activity than Samples *b* and *d*, which gave the same rate constant.

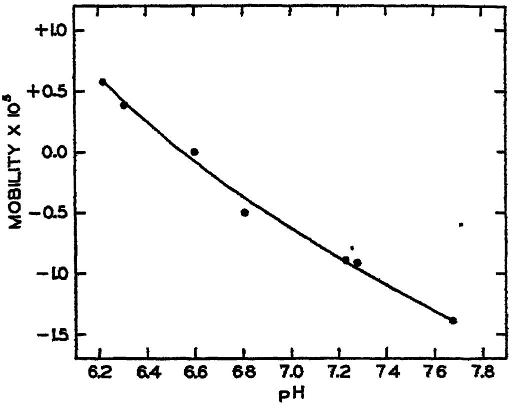

FIG. 4. Electrophoretic mobility of D-glyceraldehyde-3-phosphate dehydrogenase in sq. cm. volt $^{-1}$ sec. $^{-1}$ $\times 10^5$ at 2° in phosphate buffers of ionic strength 0.1.

The usual composition of the reaction mixture in moles per ml. was 2.5 $\times 10^{-7}$ for the D component of DL-glyceraldehyde phosphate,[3] 1 $\times 10^{-7}$

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[3] We are indebted to Dr. H. O. L. Fischer, Dr. E. Baer, and Dr. H. A. Lardy for several samples of synthetic DL-glyceraldehyde phosphate (9). Determinations of

DPN,[4] 6 × 10^{-6} arsenate, 3 × 10^{-5} pyrophosphate, 4 × 10^{-6} cysteine, pH 8.5. The reference cell contained the same reactants with the exception of the enzyme. The concentrations of triose phosphate and DPN are about 6 and 2.5 times higher, respectively, than those required to give one-half saturation of the enzyme. Bimolecular rate constants were calculated from the equation

$$K = \frac{2.3}{t(a - b)} \log \frac{b\,(a - x)}{a\,(b - x)}$$

where a is the initial concentration of triose phosphate, b that of DPN, and x the amount of reduced DPN formed in time t (minutes), all expressed in moles per ml. Reduced DPN was calculated from the spectrophotometric readings by means of the relation, $(2.3 \log I_0/I)/1.45$. This corresponds to the β coefficient for pure reduced DPN determined by Ohlmeyer (10). In some cases equal concentrations of triose phosphate and DPN were used in which case the equation reduces to

$$K = \frac{1}{t}\,\frac{x}{a(a - x)}$$

For protein determinations an aliquot of the stock solution was diluted with water and read immediately at the wave-length of maximum absorption (276 mμ) in the spectrophotometer. The conversion factor, based on micro-Kjeldahl determinations, was 1.9; hence $(2.3 \log I_0/I)/1.9$ = mg. of protein per ml. In some cases protein was determined by the biuret method of Robinson and Hogden (11) or, in the case of dialyzed enzyme solutions, according to the modification of this method by Weichselbaum (12). For comparison of specific activities, the rate constants were divided by mg. of protein present per ml. of reaction mixture.

Proportionality could be tested for only a limited range of enzyme concentrations (0.8 to 5 γ per ml.), because at higher enzyme concentrations the rate of reaction was too fast to permit accurate galvanometer readings. Within the range tested, proportionality was satisfactory. The bimolecular rate constant decreased somewhat with time. This was the case in the presence or absence of cysteine, as well as when glutathione was substituted for cysteine. For example, with 4 γ of protein per ml. the rate constants for 1, 2, and 3 minutes were 2.59, 2.49, and 2.44 × 10^6, respectively, giving a specific activity for the 1st minute of $(2.59 \times 10^6)/0.004 = 6.5 \times 10^8$ at 24°.

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alkali-labile P or of the amount of DPN reduced enzymatically were used to calculate concentrations.

[4] In most of the experiments a sample of 50 per cent purity prepared in this laboratory was used.

An example of the activating effect of cysteineis given in Fig. 5. Separate tests have shown that the effect of cysteine on the enzyme at room temperature is not instantaneous; maximum activity is reached in 5 to 7 minutes and is thereafter maintained for about 30 minutes, followed by a decline in activity after longer periods of incubation at room temperature. In order to test the enzyme under optimal conditions, it is diluted with and then kept for 7 minutes in the cysteine-pyrophosphate buffer in the presence of DPN; the reaction is then started by the addition of triose phosphate plus arsenate.

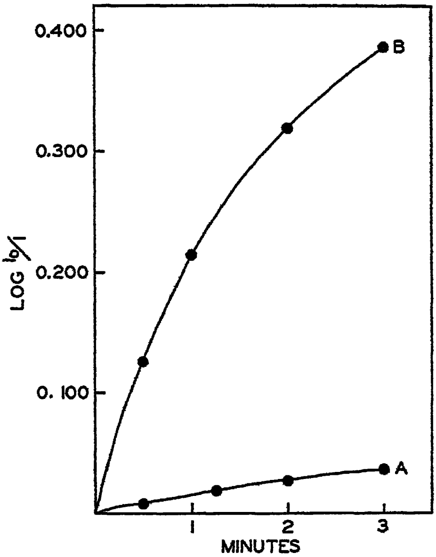

FIG. 5. Activating effect of cysteine on glyceraldehyde phosphate dehydrogenase. In Curve A, a dilute solution of the enzyme (4 γ per ml.) was kept for 10 minutes at 25° in the absence of cysteine. Curve A shows that the enzyme was practically inactive when tested in the absence of cysteine. Curve B shows that, when the same dilute solution of enzyme was pretreated with and tested in the presence of cysteine, it regained activity.

Triose phosphate reacts with cysteine, but this source of error is small because of the low concentration of cysteine (0.004 M), because triose phosphate is added last, and because the reaction is measured for only 2 minutes. When 2.5×10^{-4} M triose phosphate is incubated with 4×10^{-3} M cysteine for 6 minutes at room temperature before the enzyme is added, the loss of triose phosphate reduces the rate of the reaction by about 20 per cent. Cysteine is a disturbing factor in the measurement of the equilibrium of the reaction in the presence of phosphate. After apparent equilibrium has been reached, reduced DPN is slowly reoxidized, due to the removal of triose phosphate by cysteine. Glutathione reacts much more slowly with triose phosphate, as is shown by the fact that the equilibrium position is maintained unchanged for 10 minutes; furthermore the same equilibrium is reached in the presence and absence of glutathione. It has been found that glutathione is not as effective as cysteine in maintaining dilute enzyme solutions at the level of maximum activity; hence cysteine is preferred for rate, while glutathione is preferred for equilibrium measurements.

Stability—Enzyme crystals left suspended in 0.66 saturated $(NH_4)_2SO_4$ and stored in the refrigerator lost 50 per cent of their original activity after 2.5 months. The small amount of enzyme which remains in solution in 0.66 saturated $(NH_4)_2SO_4$, pH 8.3, is stable for at least 24 hours at 3°.

In weak salt solution, stability of the enzyme is dependent, among other factors, on pH. An enzyme solution (1 mg. per ml.) was incubated for 30 minutes at 30° in acetate buffer at pH 5.5 and in pyrophosphate buffer at pH 7.3; the loss of enzyme activity was 27 per cent in the former and 17 per cent in the latter case. More dilute enzyme solutions are inactivated more rapidly under these conditions.

It has been found, in confirmation of Rapkine (13), that DPN exerts a protective effect on dilute enzyme solutions in the absence of cysteine. For example, an enzyme solution (2.5 γ per ml.) incubated at room temperature for 10 minutes in the presence of DPN, but in the absence of cysteine, retained 85 per cent of its activity when compared with a sample incubated with cysteine in the usual way. When incubated without DPN the enzyme was almost completely inactive.

Dissociation Constants of Substrates—The concentrations of triose phosphate and DPN were varied over a 10-fold range in enzymatic tests at pH 8.5. Satisfactory straight lines were obtained when the reciprocal of concentration was plotted against the reciprocal of the log I_0/I reading at 1 minute. The values obtained from these graphs were 5.1×10^{-5} moles per liter for D-glyceraldehyde phosphate and 3.9×10^{-5} moles per liter for DPN. For the yeast enzyme, Warburg and Christian found 3.2×10^{-5} moles per liter for DPN at pH 7.5 with DL-glyceraldehyde as substrate. When recalculated by the method indicated above, the value would be about 5×10^{-5} moles per liter.

Activity at Different pH Values and Turnover Number—In these experiments, the enzyme was saturated with both glyceraldehyde phosphate and DPN[5] by using initial concentrations of each of 4.8×10^{-4} moles per liter. The initial rate of the reaction (obtained by extrapolation to zero time) was the same at pH 8.6 and 9.0; at pH 8.1, 7.7, and 7.1 it was 81, 57, and 15 per cent, respectively, of the rate obtained at the higher pH values.

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[5] A preparation of 80 per cent purity as determined by enzymatic reduction was kindly supplied by Dr. A. Kornberg.

Because of the instability of the enzyme protein, it has not been possible, so far, to obtain satisfactory values for the diffusion and sedimentation constants. The turnover number for 100,000 gm. of protein corresponds to a reduction of 6700 moles of DPN per minute at pH 8.6 and 27°. From the data given above, it may be seen that the turnover number at the pH of muscle would be considerably lower. The high concentration of this enzyme in muscle may be related to this relatively low catalytic activity.

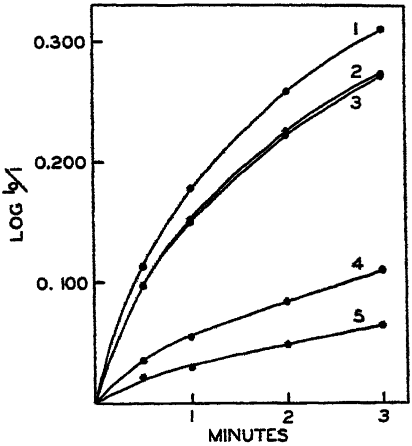

FIG. 6. Rate of inactivation of glyceraldehyde phosphate dehydrogenase by 0.0001 M iodoacetate at 0°, pH 7.1. Activity was measured at 27° at pH 8.5. Curves 1 and 2, control samples tested after being kept for 1 and 30 minutes at 0°. Curves 3, 4, and 5, samples tested after being kept for 1, 15, and 30 minutes in the presence of iodoacetate at 0°.

A twice crystallized sample of yeast glyceraldehyde phosphate dehydrogenase, prepared in this laboratory by Dr. E. G. Krebs by a modification of the method of Warburg and Christian, was tested under the same conditions as the muscle enzyme. Dr. Krebs found that the crystalline yeast enzyme requires a reducing agent such as cysteine for full activity, a fact which is not mentioned by Warburg and Christian. The turnover number of the yeast enzyme was of the same order of magnitude as that of the muscle enzyme.

Iodoacetate—The well known inhibition of lactic acid fermentation in muscle and of alcoholic fermentation in yeast has been shown to be at the triose phosphate level (14) when low concentrations of iodoacetate are used. That the triose phosphate dehydrogenase may be classified as an "—SH" enzyme has been established by the work of Rapkine and Trpinac (15–17). It has also been shown that the inhibition by iodoacetate is not instantaneous and that it cannot be reversed by cysteine. It seemed of interest to repeat some of these observations with the crystalline enzyme preparation.

The curves in Fig. 6 show the rate of inactivation of the enzyme by 10^{-4} M iodoacetate at 0° and pH 7.1. No cysteine was used in this experiment and consequently relatively large amounts of enzyme (about 100 γ) had to be used in the activity measurements, which were carried out at 27°. A control sample of the enzyme was kept at 0° in the absence of iodoacetate in order to determine the amount of spontaneous inactivation.

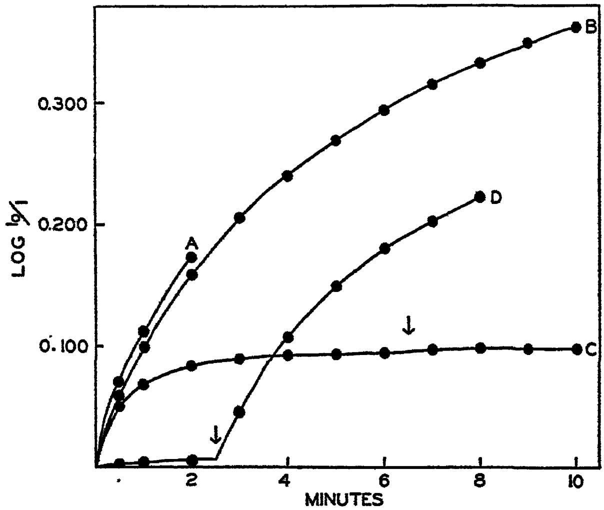

FIG. 7. Irreversibility of the iodoacetate inhibition of glyceraldehyde phosphate dehydrogenase by cysteine. The methods used for obtaining the enzyme in the reduced or oxidized form are given in the text. Curves A and B, activity of the reduced enzyme in the presence and absence of cysteine, respectively. Curve C, activity of the reduced enzyme in the presence of 0.0004 M iodoacetate; cysteine added at time indicated by the arrow. Curve D, activity of oxidized form of the enzyme; cysteine added at time indicated by arrow. Activity was measured at 23° at pH 8.5.

The enzyme lost 15 per cent of its activity in 30 minutes without iodoacetate and 15, 70, and 85 per cent in the presence of iodoacetate after incubation periods of 1, 15, and 30 minutes.

In the above experiment only a small part of the enzyme was in the active or "reduced" form. This is shown by the fact that the enzyme had only about 10 per cent of the activity it had when tested in the presence of cysteine. The inactive or "oxidized" form of the enzyme does not react with low concentrations of iodoacetate and is converted to the reduced form by cysteine; this simulates a reversal of the iodoacetate inhibition by cysteine.

In Fig. 7 are shown the effect of iodoacetate on a fully reduced enzyme and the non-reversibility of the inhibition by cysteine. A solution of 2 mg. of enzyme per ml. in 0.03 M cysteine-pyrophosphate buffer, pH 8.5, was kept for 15 minutes at 24° in order to reduce the enzyme. This solution was then stored at 0° and aliquots were diluted 30-fold with 0.004 M cysteine-pyrophosphate as needed. As soon as each dilution was made 0.1 ml. of the dilution (7 γ of enzyme) was treated as follows. In Curve A, 0.1 ml. was incubated for 7 minutes in 0.004 M cysteine-pyrophosphate pH 8.5, and triose phosphate was added to start the reaction; in Curve B, 0.1 ml. was added to the otherwise complete, but cysteine-free reaction mixture, the rate of reaction in Curves A and B being nearly the same; in Curve C, 0.1 ml. was added to start the reaction in the same way as for Curve B, except that the reaction mixture contained iodoacetate. Curve C shows that even at 23° the inhibition by iodoacetate (final concentration 4×10^{-4} M) requires several minutes for completion and that addition of cysteine, at a time at which the reaction has practically stopped, did not remove the inhibition. In contrast to this is the reactivation by cysteine of the oxidized form of the enzyme, Curve D. In Curve D the enzyme (70 γ per ml.) had been allowed to remain at 22° in the absence of cysteine for 30 minutes. The initial rate in the absence of cysteine was insignificant, but the addition of cysteine rapidly reestablished activity.

The authors wish to thank Mr. Robert Loeffel and Dr. A. A. Green for carrying out the measurements of electrophoretic mobility reported in this paper.

SUMMARY

1. The enzyme D-glyceraldehyde-3-phosphate dehydrogenase has been isolated and crystallized by a method involving fractionation with ammoniacal $(NH_4)_2SO_4$ solution of an extract of rabbit muscle prepared with dilute alkali. The enzyme crystallizes from a dilute solution (about 0.1 per cent) of purity level of about 0.5, when the saturation with $(NH_4)_2SO_4$ is 0.72 and the pH 8.2 to 8.4. 1 gm. or more of crystalline enzyme is obtained from 500 gm. of muscle. On recrystallization from more concentrated enzyme solutions at 0.66 saturation with $(NH_4)_2SO_4$ only a slight gain in specific enzyme activity results.

2. Aldolase and the dehydrogenase can be prepared from the same muscle extract.

3. The recrystallized enzyme is electrophoretically homogeneous over the pH range 6.2 to 7.7. In phosphate buffer, ionic strength 0.1, the isoelectric point is at pH 6.55.

4. Activity of the enzyme was measured spectrophotometrically accord-

ing to the method of Warburg and Christian. In order to obtain maximum activity the enzyme has to be diluted and preincubated in a cysteine (or glutathione) solution.

5. In weak salt solution the enzyme has highest stability around neutrality. At pH 5.2 or 9 it is rapidly denatured even at 0°.

6. The enzyme has one-half maximum activity when the concentration of glyceraldehyde-phosphate is 5.1×10^{-5} moles per liter. The corresponding value of DPN is 3.9×10^{-5} moles per liter.

7. The enzyme activity is highest between pH 8.6 and 9 and drops off sharply on the acid side, so that at pH 7.1 it is only 15 per cent of the maximum rate.

8. Iodoacetate in low concentration (10^{-4} M) inhibits the enzyme. This inhibition, even at 27°, is not instantaneous. Cysteine does not reverse the inhibition.

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THE PROSTHETIC GROUP OF CRYSTALLINE D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*

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It is known that the major portion of the ultraviolet absorption of proteins in the region 250 to 300 mμ is due to the presence of the aromatic amino acids, chiefly tyrosine and tryptophan. The ultraviolet absorption spectrum of D-glyceraldehyde-3-phosphate dehydrogenase differs appreciably from the spectrum calculated from the chemically determined tyrosine and tryptophan contents (1) both at neutral and alkaline pH, Figs. 1 and 2. At neutral pH this difference is particularly noticeable at 250 mμ. At pH 12 to 13 the absorption maximum of tyrosine plus tryptophan shifts from about 280 to about 290 mμ. The dehydrogenase exhibits this spectral shift at pH 12 but shows only a barely detectable minimum at 278 mμ. This property indicates the presence of groups which absorb in the 260 to 280 mμ region, and which are not grossly affected by changes in pH. Such behavior is shown by the C = N bond as it occurs in purines and pyrimidines. We were therefore led to suspect the presence of nucleotides in the recrystallized protein. This idea was supported by the observation that a considerable amount of humin was formed during acid hydrolysis, indicating the presence of carbohydrate, and that the exhaustively dialyzed enzyme contained 0.126 per cent of phosphorus.

The most likely substance to be looked for was diphosphopyridine nucleotide (DPN), since it participates in the reaction catalyzed by the enzyme. It proved possible to demonstrate that the crystalline enzyme contains a constant amount of DPN in rather firm combination.

The presence of DPN in the enzyme is indicated by the following data.

1. The characteristic absorption maximum of reduced DPN at 340 mμ appears when glyceraldehyde phosphate and arsenate are added to a 0.77 per cent solution of the enzyme, Fig. 3. The absorption observed at 340 mμ corresponds to 0.6 mole of DPN in 50,000 gm. of the dehydrogenase. This band disappears again when pyruvate and lactic dehydrogenase (a purified fraction from rabbit skeletal muscle) are added.

2. The filtrate obtained by precipitating a suitable amount of enzyme

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* This work was supported in part by a grant from the Nutrition Foundation, Inc.

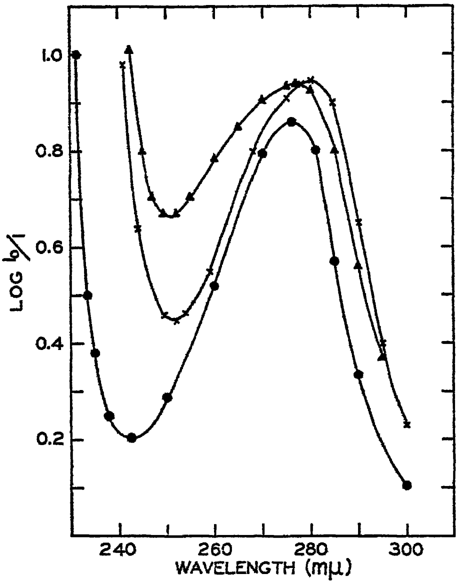

FIG. 1. Absorption spectrum of glyceraldehyde phosphate dehydrogenase, 1 mg. per ml., at neutral pH. ▲, untreated enzyme; ×, enzyme after treatment with phosphatase and precipitation with (NH$_4$)$_2$SO$_4$, 63 per cent of the DPN having been removed (Experiment 3, Table II); ●, calculated absorption due to tyrosine and tryptophan present.

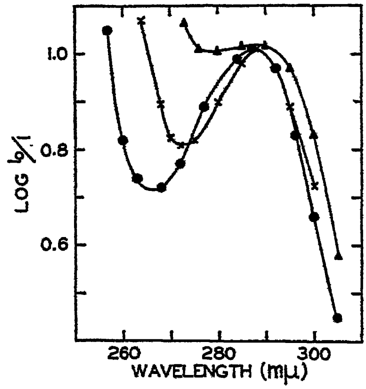

FIG. 2. Absorption spectrum of glyceraldehyde phosphate dehydrogenase, 1 mg. per ml. in 0.1 N NaOH. ▲, untreated enzyme; ×, enzyme after treatment with phosphatase and precipitation with (NH$_4$)$_2$SO$_4$ (Experiment 3, Table II); ●, calculated absorption due to tyrosine and tryptophan present.

with trichloroacetic acid can replace DPN in specific enzymatic tests, Fig. 4. The absorption observed at 340 mμ after enzymatic reduction of the trichloroacetic acid filtrate corresponds to 0.8 mole of DPN split off from 50,000 gm. of the dehydrogenase.

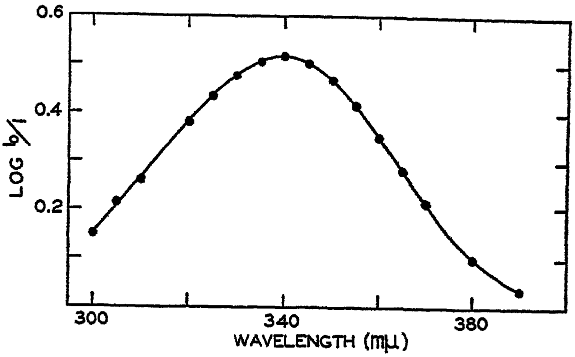

FIG. 3. Appearance of the reduced band of DPN at 340 mμ in a solution containing 7.7 mg. of enzyme protein per ml. after addition of arsenate and excess glyceraldehyde phosphate. The reference cell contained the same solution with the exception of glyceraldehyde phosphate.

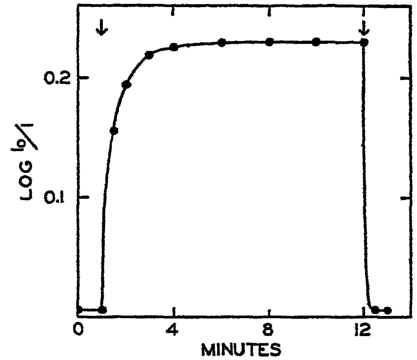

FIG. 4. Demonstration of DPN in the trichloroacetic acid filtrate of glyceraldehyde phosphate dehydrogenase. Neutralized filtrate, corresponding to 5.77 mg. of enzyme protein, + cysteine-pyrophosphate buffer + arsenate + glyceraldehyde phosphate in 3 ml., measured against the control containing neutralized trichloroacetic acid instead of the filtrate. The reaction started at the first arrow by addition of 6 γ of glyceraldehyde phosphate dehydrogenase per ml. At the second arrow a solution containing pyruvate + purified lactic dehydrogenase was added to both vessels.

3. The enzyme contains adenine, nicotinamide, ribose, and phosphate in the ratios in which these substances are present in DPN. Table I summarizes the data obtained for the four constituents of DPN.

Adenine and nicotinamide were determined microbiologically.[1] A sample of the exhaustively dialyzed and dried enzyme was hydrolyzed at a bath temperature of 120° for 1 hour in a mixture of formic acid (specific gravity 1.2), concentrated hydrochloric acid, and water in the volume ratios of 5 :1 :4. This procedure liberates the purines from nucleotides without appreciable decomposition (2). The hydrolysate was repeatedly evaporated to dryness *in vacuo*, then neutralized to pH 6 and diluted to volume.

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[1] We are indebted to Dr. Ethel Ronzoni and Mr. Lester Wicks for the assay of nicotinamide and adenine, respectively.

Assay for adenine was carried out with the adenineless mutant of *Neurospora crassa* 28610 by the procedure described by Mitchell and Houlahan (3). The cultures were grown in 50 ml. flasks and the standard curves covered the range of 0 to 200 γ of adenine. The growth response indicated the presence of 0.25 ± 0.04 per cent[2] adenine in the enzyme.

TABLE I

Analysis of Crystalline Glyceraldehyde Phosphate Dehydrogenase for Components of Diphosphopyridine Nucleotide

Substance	Source	Per mg. of enzyme	Substance in 50,000 gm. enzyme
		γ	*moles*
Adenine	Hydrolysate of dialyzed, dried enzyme	2.5	0.9
Nicotinamide	" " " " "	2.5	1.0
Phosphorus	Dialyzed, dried enzyme, ashed	1.26	2.0
	Trichloroacetic acid filtrate of native enzyme	1.23	2.0
Ribose	Native enzyme	5.90	1.97
	Dialyzed, dried enzyme	5.83	1.95

Nicotinic acid was determined in similar hydrolysates with *Lactobacillus arabinosus* according to the procedure of Snell and Wright (4). This organism was chosen because it shows equal responses to nicotinic acid and nicotinamide, and hence no appreciable error was introduced by incomplete hydrolysis of the amide. The growth response indicated the presence of 0.25 ± 0.02 per cent[2] nicotinamide in the enzyme.

Phosphate was determined by the Fiske and Subbarow method (5). The color was developed in glass-stoppered tubes, graduated at 1 ml., and was read at 660 mμ in the Beckman photoelectric spectrophotometer, equipped with the microadaptation described by Lowry (6). In this manner 1 γ of phosphorus per ml. could be determined accurately. Ashing of the trichloroacetic acid filtrates was carried out in the same tubes in which the color was developed.

Ribose was determined by the orcinol reaction (7). Freshly recrystallized orcinol was dissolved in 95 per cent alcohol (50 mg. per ml.);

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[2] Average deviation.

this solution is stable for a few days when kept in an amber bottle in the cold. 2 ml. of 0.04 per cent ferric chloride in concentrated HCl, 2 ml. of unknown (or of standard) and 0.6 ml. of the alcoholic orcinol solution were mixed in a Klett colorimeter tube, covered, and heated for 35 minutes at 100°. The peak of absorption of the resulting green color, as determined in the Beckman spectrophotometer, was at 670 mμ. Readings in this instrument at this wave-length or in the Klett-Summerson colorimeter with Filter 660 gave concordant results.

According to Albaum and Umbreit (8), both free ribose and yeast adenylic acid (3-phosphoriboside) show a much slower rate of color development than does muscle adenylic acid (5-phosphoriboside). The pentose in the enzyme showed the same rate of color development as free ribose; *e.g.*, the ratio of the densities at 660 mμ after 8 and 35 minutes of heating was 2.2 for free ribose, 2.15 for the pentose in the enzyme, and 1.29 for muscle adenylic acid. It was therefore of interest to test DPN; although the sample was not pure, it showed a rate of color development similar to that of free ribose.[3] Since pure DPN was not available, free ribose was used as the standard.

Pentose determinations were carried out on 1 to 2 mg. of enzyme, either on the protein directly (the solution becomes clear during heating) or on a trichloroacetic acid filtrate. That the DPN which is present in the enzyme appears in the trichloroacetic acid filtrate has been shown in Fig. 4. Pentose determinations on the protein directly and on the trichloroacetic acid filtrate showed that all of the pentose (and phosphate) appeared in the filtrate. A less complete recovery of pentose was obtained in the filtrate of a heat-coagulated enzyme solution.

The average of ten determinations, made on several repeatedly recrystallized enzyme preparations, was 0.59 ± 0.04 per cent[2] of pentose. In these determinations the enzyme crystals suspended in 0.66 saturated $(NH_4)_2SO_4$ were centrifuged down at high speed in the cold, and dissolved in water. Aliquots were then used for pentose and for spectrophotometric protein determinations.

As shown in Table I, the same analytical values were obtained on enzyme preparations that had been exhaustively dialyzed against distilled water as on undialyzed preparations. When the enzyme was dialyzed at pH 5.3 or 9.0 overnight at 0°, it lost most of its activity, but retained its DPN as shown by pentose determinations. When a solution of the enzyme was crystallized in the presence of added DPN (about 10 times the amount present in the enzyme) and then recrystallized twice, its pentose content was no higher than that of the original enzyme.

[3] This indicates, incidentally, that the rate of color development is not a reliable criterion for the differentiation of 5- and 3-phosphoribosides.

These experiments show that the DPN is rather firmly combined with the enzyme and that there is a stoichiometric ratio between protein and DPN. The analytical results in Table I indicate that the minimum molecular weight of the protein, based on its DPN content, is 50,000. According to the amino acid data (1), the minimum molecular weight is larger than this by a multiple of 2. Direct spectrophotometric estimation carried out both on the intact enzyme and on a trichloroacetic acid filtrate prepared from it indicates that at least 0.6 mole and 0.8 mole of DPN, respectively, are present in 50,000 gm. of the enzyme protein (Figs. 3 and 4).

4. Treatment of the enzyme with phosphatase or norit removes DPN. In both cases the catalytic activity of the enzyme is preserved (Table II), but the enzyme can no longer be crystallized from 0.66 saturated $(NH_4)_2SO_4$.

TABLE II

Effect of Treatment with Phosphatase or Norit on Pentose Content of Glyceraldehyde Phosphate Dehydrogenase

Experiment No.	Enzyme treated with	Pentose per mg. enzyme		Activity of treated enzyme as per cent of control enzyme
		Control	Treated	
		γ	γ	
1	Phosphatase	6.46	1.35	95
2	"	5.64	0.41	67*
3	"	6.31	2.34	92 (96†)
4	Norit	6.50	1.36	86
5	"	6.80	0.65‡	
6	"	6.80	2.43	
			1.09‡	

* Incubated without cysteine.

† After precipitation with $(NH_4)_2SO_4$.

‡ After two norit treatments.

In the phosphatase experiments, solutions of the enzyme (15 to 30 mg. per ml.) were incubated 15 to 30 minutes at pH 7.9 to 8.5 at 30° in the presence of 0.1 mg. of phosphatase protein[4] per 25 to 50 mg. of dehydrogenase. In two of the three experiments, 0.006 M cysteine was present during incubation to stabilize the dehydrogenase. Control samples without phosphatase were treated in the same way. At the end of the incubation period, enzymatic activity was determined and the rest of the solution was brought to 0.66 saturation with $(NH_4)_2SO_4$ in a manner designed to effect crystallization of the dehydrogenase. In one instance an amount of phosphatase equivalent to that used during incubation was added to the control sample after salt addition. While crystals formed overnight in the control samples, none formed even after prolonged standing in the samples which had been incubated with phosphatase. Both the crystalline precipitate of the controls and the amorphous precipitate of the experimental samples were separated by centrifugation, washed with 0.7 saturated $(NH_4)_2SO_4$, and the pentose content of the protein determined. Table II shows that the treated enzyme had lost a considerable part of its pentose, while its enzymatic activity was not destroyed. That DPN was split by the phosphatase was shown by the liberation of inorganic phosphate. In Experiment 3, enzymatic activity was also determined after precipitation with $(NH_4)_2SO_4$.

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[4] We are indebted to Dr. Gerhard Schmidt for the sample of purified intestinal phosphatase.

In Figs. 1 and 2, absorption spectra of the phosphatase-treated enzyme are presented. The removal of DPN is shown by a marked decrease of absorption between 245 and 265 mμ at neutral pH, and between 265 and 285 mμ at pH 12. The enzyme used in the absorption measurements was that of Experiment 3, Table II, in which 63 per cent of the DPN had been removed (on the basis of pentose determinations).

In the norit experiments, the enzyme crystals were separated by centrifugation and dissolved in ice-cold water to give a 1 to 3 per cent protein solution. A suspension of norit was added, so that the mixture contained 13 mg. of norit per ml. After gentle agitation for 3 minutes in the cold room the norit was removed by centrifugation and filtration. The loss of protein was 10 to 15 per cent. After treatment with norit, protein was determined by the method of Robinson and Hogden (9). By repetition of the norit treatment it was possible to reduce the pentose content of the enzyme to 0.65 γ per mg. The treated enzyme was brought to 0.66 saturation with $(NH_4)_2SO_4$ solution, pH 8.4, one aliquot directly and one after addition of DPN (0.23 micromole per mg. of protein, about 10 times the amount present in the enzyme). In the former case an amorphous precipitate formed and slowly settled. In the latter case, crystals formed rapidly. These crystals were indistinguishable from those of the original enzyme preparation. After two recrystallizations their pentose content was 6.85 γ per mg. of protein. This is within the range of values found with untreated enzyme preparations. These experiments show that the enzyme can recombine with DPN and that it crystallizes as a protein-DPN complex. Furthermore, in this recombination the original ratio of protein to DPN is reestablished.

SUMMARY

1. Crystalline D-glyceraldehyde-3-phosphate dehydrogenase from rabbit skeletal muscle contains a definite amount of firmly bound diphosphopy-

ridine nucleotide (DPN). The minimum molecular weight of the enzyme calculated from its DPN content is 50,000.

2. The presence of DPN is shown by the following data. (*a*) The ultraviolet absorption spectrum of the enzyme indicates the presence of groups other than tyrosine or tryptophan in the region 250 to 280 mμ. (*b*) The addition of D-glyceraldehyde phosphate and arsenate to a concentrated solution of the enzyme leads to the appearance of the characteristic absorption maximum of reduced DPN. The band at 340 mμ disappears when pyruvate and lactic dehydrogenase are added. (*c*) Precipitation by heat or trichloroacetic acid liberates a substance in the filtrate that can replace DPN in dehydrogenase test systems. (*d*) Analyses show that the enzyme contains adenine, nicotinamide, phosphorus, and ribose in the ratio 1:1:2:2.

3. Prolonged dialysis of the enzyme between pH 5.3 and 9.0 does not remove the nucleotide. The DPN may be removed by treatment with intestinal phosphatase or norit; after such treatment the protein retains its activity but does not crystallize. The addition of DPN (after norit treatment) leads to the formation of crystals containing the original ratio of DPN to protein. From this it is concluded that the enzyme crystallizes as a protein-DPN complex.

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THE AMINO ACID COMPOSITION OF ALDOLASE AND D-GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE

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Aldolase (1) and D-glyceraldehyde-3-phosphate dehydrogenase (2) catalyze consecutive steps in the series of enzymatic reactions that lead to the formation of lactic acid. These enzymes which constitute about 10 and 7 per cent respectively of the water-soluble proteins of rabbit skeletal muscle show significant differences in solubility, isoelectric point, stability in solution, sulfhydryl sensitivity, and crystal form. The amino acid analyses to be described account, within the limits of error of the methods, for essentially all of the nitrogen in the two proteins. Small but significant differences exist in the proportions of all but a few amino acids and at least four of the amino acids occur in the two proteins with widely differing frequencies. In spite of the differences, there are elements of similarity in the composition of the two enzymes, particularly the low contents of glutamic acid, which differentiate them from the structural proteins of muscle.

The analysis of aldolase is summarized in Table I. Amino acid residue numbers are calculated on the basis of a minimum molecular weight of 140,000. This number is calculated from the molar ratios of the six amino acids present in smallest amounts, as listed in Table II. The result falls between the values 136,000 and 150,000 determined respectively by sedimentation equilibrium and by sedimentation velocity and diffusion on myogen A (3).

Table III contains a summary of the amino acid analyses of the dehydrogenase. In this table the residue numbers are calculated on the basis of a minimum molecular weight of 99,060. The calculations of minimum molecular weight, based upon chemically determined cystine, tyrosine, tryptophan, proline, and phosphorus, and a microbiological methionine determination, are shown in Table IV. The value 99,060 is very close to twice the minimum molecular weight calculated from the content of diphosphopyridine nucleotide (4).

The nitrogen content of the proteins calculated from their amino acid composition is slightly higher than the total nitrogen determined by the micro-Kjeldahl procedure, Tables I and III. However, the differences fall within the limits of possible cumulative error in the summation of twenty independently determined quantities. It is not possible to state with certainty that the balances as obtained exclude the presence of undetected nitrogenous components, although the presence of such substances appears unlikely.

TABLE I

Amino Acid Composition of Crystallized Aldolase from Rabbit Skeletal Muscle

Amino acid	Gm. per 100 gm. protein; average deviations	Gm. nitrogen per 100 gm. protein	No. of residues per 140,000 gm.	Methods
Glycine	5.61	1.047	104.7	ID
Alanine	8.56	1.346	134.6	"
Valine	7.40 ± 0.1	0.885	88.5	LM, LA
Leucine	11.5 ± 0.2	1.228	122.8	LA
Isoleucine	7.87 ± 0.4	0.840	84.0	"
Half cystine	1.12 ± 0.03	0.131	13.1	Chem[1]
Methionine	1.17 ± 0.2	0.110	11.0	LM, LF
Serine*	6.57 ± 0.1	0.876	87.6	" Chem[2]
Threonine*	7.1 ± 0.4			Chem[3]
	6.5 ± 0.3	0.764	76.4	LF
Arginine	6.33 ± 0.2	2.037	50.9	LM, Chem[4]
Histidine	4.21 ± 0.2	1.141	38.0	"
Lysine	9.54 ± 0.2	1.829	91.4	"
Proline	5.71	0.695	69.4	ID
Phenylalanine	3.06 ± 0.1	0.260	26.0	LM
Tyrosine	5.31	0.411	41.1	Chem[5]
Tryptophan	2.31	0.317	15.9	Chem[6, 7]
Aspartic acid	9.7 ± 0.2	1.020	102.1	LM
Glutamic "	11.4 ± 0.2	1.085	108.8	"
Amide nitrogen		0.91	91	Chem[8]
Total	114	16.9		
" nitrogen		16.8		Kjeldahl

Key to methods: ID isotope dilution (pipsyl derivative), LM bioassay (*Leuconostoc mesenteroides*), LA bioassay (*Lactobacillus arabinosus*), LF bioassay (*Lactobacillus fermenti*), Chem[1] photometric (phosphotungstic acid), Chem[2] photometric (periodate, chromotropic acid), Chem[3] photometric (periodate, *p*-hydroxydiphenyl), Chem[4] photometric (hypobromite), Chem[5] photometric (mercuric chloride, nitrous acid), Chem[6] photometric (ultraviolet on mercury precipitate), Chem[7] photometric (*p*-dimethylaminobenzaldehyde), Chem[8] titrimetric (micro diffusion).

* We have not corrected the serine and threonine values for destruction during hydrolysis.

Both proteins are relatively high in lysine and have similar but not identical contents of arginine and histidine. Aldolase is higher by about a factor of 2 in glutamic acid and leucine and is considerably lower than the

TABLE II

*Minimum Molecular Weight of Aldolase**

Amino acid	Gm. per 100 gm. protein	Molar ratio										
Methionine	1.17	1	2	3	4	5	6	7	8	9	10	11
Half cystine	1.12	1.188	2.38	3.56	4.75	5.94	7.13	1.11	9.50	10.7	11.9	13.1
Tryptophan	2.31	1.447	2.95	4.34	5.79	7.23	8.68	10.1	11.5	13.0	14.5	15.9
Phenylalanine	3.06	2.363	4.72	7.09	9.45	11.8	14.2	16.5	18.9	21.3	23.6	26.0
Histidine	4.21	3.455	6.91	10.4	13.8	17.3	20.7	24.2	27.6	31.1	34.5	38.0
Tyrosine	5.31	3.373	7.47	11.3	14.9	18.7	22.4	23.6	29.9	33.6	37.4	41.1
Mol. wt.		12,735	25,470	38,205	50,940	63,675	76,410	89,145	100,880	114,615	127,350	140,085
No. of integral ratios		1	3	2	2	2	2	2	3	3	2	6

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* The complete homogeneity of this protein has not yet been satisfactorily established by physical methods, although the available evidence is favorable (1). For the calculation of a minimum molecular weight of 140,000 to be accepted with confidence, the individual analyses must have an accuracy of ±1 per cent. We have not been able to supply independent evidence that the above analyses are actually accurate to within these limits. The above calculations are offered as one test of consistency of the data.

dehydrogenase in its content of valine and phenylalanine. The glutamic acid level in the dehydrogenase is among the lowest ever recorded for a protein and the valine level is among the highest. Serine and threonine are similar in the two proteins. The cystine contents are similar and low and do not reflect the apparent differences in sulfhydryl sensitivity.

TABLE III

Amino Acid Composition of D*-Glyceraldehyde Phosphate Dehydrogenase from Rabbit Skeletal Muscle*

Amino acid	Gm. per 100 gm. protein with average deviations	Gm. nitrogen per 100 gm. protein	No. of residues per 99,100 gm.	Methods*
Glycine	6.03	1.125	79.6	ID
	6.15 ± 0.1			LM
Alanine	6.72	1.058	74.7	ID
Valine	12.0 ± 0.1	1.483	104.9	LM, LA
Leucine	6.78 ± 0.1	0.724	51.2	" "
Isoleucine	9.1 ± 0.3	0.972	68.7	LA
Half cystine	1.09	0.127	9.0	Chem[1]
Methionine	2.70 ± 0.2	0.253	18.0	LM, LF
Serine†	6.7 ± 0.1	0.893	63.2	"
	7.7			Chem[2]
Threonine†	6.9 ± 0.2	0.811	57.4	LF
	7.2 ± 0.5			Chem[3]
Arginine	5.23 ± 0.1	1.682	29.8	LM, Chem[4]
Histidine	5.01 ± 0.1	1.357	32.0	"
Lysine	9.42 ± 0.3	1.804	63.9	"
Proline	3.67 ± 0.03	0.446	31.6	ID
Phenylalanine	5.55 ± 0.06	0.471	33.3	LM
Tyrosine	4.57	0.353	25.0	Chem[5]
Tryptophan	2.05	0.281	9.9	Chem[6, 7]
Aspartic acid	12.4 ± 0.2	1.304	93.2	LM
Glutamic "	6.8 ± 0.2	0.647	45.8	LM, LA
Amide nitrogen		0.95	67.2	Chem[8]
Diphosphopyridine nucleotide		0.20	2.0	
Total.................		16.9		
" nitrogen....... ..		16.4		Kjeldahl

* For the key to the methods, see Table I.

† See the footnote, Table I.

In comparison with the myosin fraction of muscle, data for which have been compiled by Bailey (5), glutamic acid in both enzymes is very low, histidine, serine, threonine, and valine quite high.

TABLE IV

Minimum Molecular Weight of Crystalline D-*Glyceraldehyde Phosphate Dehydrogenase from Rabbit Skeletal Muscle**

Amino acid	Gm. per 100 gm. protein	Molar ratio								
Half cystine	1.09	1	2	3	4	5	6	7	8	9
Tyrosine	4.57	2.777	5.55	8.33	11.11	22.85	16.66	19.44	36.6	25.0
Tryptophan	2.05	1.105	2.21	3.31	4.42	10.25	6.63	7.74	16.4	9.95
Methionine	2.70	1.996	3.992	5.988	7.98	9.98	11.98	13.97	15.97	17.96
Proline	3.67	3.510	7.020	10.53	14.04	17.55	21.06	24.57	28.08	31.6
Phosphorus	0.126	0.447	0.894	1.34	1.79	2.23	2.68	3.13	3.58	4.02
Mol. wt.		11,007	22,014	33,021	44,028	55,035	66,042	77,050	88,056	99,060
No. of integral ratios		2	3	2	3	2	3	2	3	5

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* The physical evidence of homogeneity of this protein is not conclusive and we therefore wish to apply here also the comments appended to Table II.

EXPERIMENTAL

The enzymes[1] were dialyzed at 4° first against running tap water, then against frequent changes of distilled water for several days. The contents of the dialysis sacs were then frozen and dried *in vacuo*. After equilibrating with the atmosphere, the moisture contents were determined by drying samples at 0.01 mm. pressure over P_2O_5 at 112°. The ash in the dried enzymes was of the order of 0.1 to 0.2 per cent. Total nitrogen was determined by the micro-Kjeldahl procedure of Miller and Houghton (6). Amide nitrogen was liberated by 2 hour hydrolysis with 2 N hydrochloric acid at 100° and determined by titration after distillation in Conway micro diffusion vessels (7). The latter analyses were carried out on dialyzed solutions of the enzymes analyzed for total nitrogen and corrected for small amounts of residual ammonium salts.

Lactobacillus arabinosus 17-5 (No. 8014) was used for the determination of glutamic acid, cystine, isoleucine, leucine, and valine. The basic medium was that of McMahan and Snell (8). It was found that omitting proline and hydroxyproline left a medium supporting normal growth and acid production, and these amino acids were therefore omitted from the media in most of the assays with this organism.

Following the study of the amino acid requirements of *Leuconostoc mesenteroides* P-60 (No. 8042) by Dunn, Shankman, Camien, Frankl, and Rockland (9), this organism was used to check the results obtained with *Lactobacillus arabinosus* and for the determination of the other amino acids essential for its growth. For histidine (10), lysine (11), and glycine (12) the methods described by Dunn and coworkers were used. The basic medium, D, of Dunn *et al.* (9) in double concentration was found suitable for the determination of the other amino acids. When the complete medium was used, addition of the maximum amount of protein hydrolysate employed in an assay caused no further increase in acid production over that given by the basal medium alone. From this it seemed probable that nonspecific stimulation effects were absent in the assays of the individual amino acids.

We followed the procedure of analyzing numerous small hydrolysates of independent enzyme preparations. Weighed samples of 20 to 100 mg. corrected for moisture were hydrolyzed for 16 hours in 6 N hydrochloric acid at a bath temperature of 125°. The hydrolysates were neutralized to pH 6 and diluted to volume. Growth was measured titrimetrically. The assay curves contained four to six points of a dilution series falling in a sensitive region of the standard curve. An analysis as reported consisted of three to seven independent assay curves. In Table V are listed the concentration ranges and conditions under which the various assays were carried out. In Table VI are shown the results of some single assay curves obtained on different preparations of the two enzymes.

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[1] These preparations were made by Dr. G. T. Cori and consisted of four to six times recrystallized samples of the enzymes.

In the course of the work, four preparations of each enzyme were used and each amino acid was determined in two or more preparations. The observed deviations between different preparations fall within the limits of variation obtained on the same preparation at different times. The average deviation expressed in Tables I and II represents unweighted averages of all analyses. Only a few obviously aberrant results were rejected. In general the errors seemed to be largely of a random nature.

TABLE V

Summary of Bioassay Conditions

Amino acid	Configuration of standard amino acid	Organism	Test volume	Range	Incubation time (37°)
			ml.	*γ per tube*	*hrs.*
Arginine	L	LM*	5	6–30	48–72
Aspartic	L	"	5	10–60	48–72
Cystine	L	" LA	5	4–20	48–72
Glutamic	L	" "	5	8–80	48–72
Glycine		"	3	8–40	72
Histidine	L	"	3	2–12	72
Isoleucine	DL	" LA	5	5–40	48–72
Leucine	DL, L	" "	5	10–50	48–72
Lysine	L	"	5	10–70	72
Methionine	DL	"	3	2–12	72
Phenylalanine	DL	"	5	4–20	72
Proline	L	"	5	6–60	48
Serine	DL	"	5	6–60	48
Tyrosine	L	"	5	3–15	72
Valine	DL	" LA	5	8–40	48–72
Threonine	DL	LF	3	7–35	48
Methionine	DL	"	3	4–20	48

* See Table I for an explanation of the contractions.

Tyrosine, tryptophan, serine, threonine, cystine, arginine, and alanine were determined chemically. The procedure of Lugg (13) with alkaline hydrolysates was followed in essential detail in the determination of tyrosine, except that the colors were read in the Beckman spectrophotometer. The tryptophan mercury complex obtained by Lugg's precipitation procedure was redissolved and determined directly without color development by its absorption in the ultraviolet as suggested by Brand and Saidel (14).

Tryptophan in the dehydrogenase was determined also on the unhydrolyzed protein as described by Sullivan and Hess (15), with results identical with those from the mercury precipitation procedure. It is of considerable interest from the standpoint of protein structure that aldolase by the same direct procedure on the unhydrolyzed protein develops the tryptophan color with *p*-dimethylaminobenzaldehyde much more slowly than other proteins and does not reach the value obtained by the isolation method.

Serine and threonine were determined by periodate oxidation. In the case of serine the formaldehyde produced in the periodate reaction was distilled from the reaction mixture as described by Boyd and Logan (16), but on a somewhat reduced scale, and determined photometrically according to the conditions defined by MacFadyen (17). The acetaldehyde resulting from the threonine oxidation was determined by the photometric method of Block and Bolling (18). In these as well as in the other chemical methods that were employed the standard solutions were carried through the same procedures as the unknowns.

TABLE VI

Results of Some Single Amino Acid Assay Curves on Different Preparations of Aldolase and D*-Glyceraldehyde Phosphate Dehydrogenase*

Enzyme	Preparation No.	Hydrolysate	Phenylalanine	Glutamic acid	Lysine	Leucine	Valine
Aldolase	1	*a*	3.17	11.6	8.3	11.6	7.36
		b		11.4			7.54
	2	*a*	3.06	11.3	8.6	11.8	7.24
		b	3.07		8.9		
	3	*a*		11.4	8.4	11.3	7.46
Dehydrogenase	1	"	5.51	7.0	9.4	6.56	12.3
		b		6.7	9.2	6.70	11.0
	2	*a*	5.56		9.8	6.57	11.9
		b	5.45				
	3	*a*		6.9	9.4	6.71	11.9

Recent investigations of Dunn, Shankman, Camien, and Block (19) with a highly enriched medium show that proline and serine are not essential for *Leuconostoc mesenteroides*. However, using Medium D, we could assay these amino acids with apparent success. The acid production in the blanks was low and there was little increase in the interval between 48 and 72 hours. The data for individual analyses at different levels show good agreement. Microbiological and chemical analyses for serine in aldolase agree to within 3 per cent. The chemical determination of serine in the dehydrogenase yielded a value of 7.69 per cent in contrast to a value of 6.6 per cent obtained by the microbiological procedure. Hydroxylysine is determined together with serine when the latter amino acid is determined as formaldehyde in the periodate reaction (20). Hydroxyproline has likewise been found to yield formaldehyde under these conditions (21). In contrast, the microbiological assay for serine is certainly independent of hydroxyproline and it is unlikely that it responds to hydroxylysine. Consequently the agreement of chemical and microbiological assays of serine in aldolase is presumptive evidence of the absence of the interfering amino acids in that protein. The possibility of their presence in the dehydrogenase must be left open, since the chemical results were definitely high.

In addition to the determination of threonine by the periodate method, which was rather variable in our hands, we have carried out microbiological assays with *Lactobacillus fermenti*. This organism in a medium deficient only in threonine slowly acquires the ability to grow without it. However, the presence of low concentrations of threonine inhibits adaptation and the organism may therefore be used for assay (22). Our results with *Lactobacillus fermenti* were consistent but were lower than the chemical determinations.

Analyses for cystine, carried out by the polarographic method of Stern, Beach, and Macy (23), established the relative levels but suffered from the uncertainties involved in the required extrapolation to zero protein concentration. The results of microbiological assays with *Leuconostoc mesenteroides* and *Lactobacillus arabinosus* were in essential agreement with the polarographic determinations but showed in individual analyses somewhat larger average variation from the mean than was desired. We therefore also analyzed for total cystine by the photometric method of Kassell and Brand (24). This procedure gave highly reproducible results about 5 per cent higher than those by the above methods.

Difficulties were encountered in the determination of alanine. While this amino acid is not absolutely essential for *Leuconostoc mesenteroides*, *Lactobacillus fermenti*, or *Streptococcus faecalis*, it does have a distinct accelerating effect on growth. Attempts to utilize this effect for assay purposes were without success and we therefore employed the chemical method of Block, Bolling, and Webb ((18) p. 266). This method has serious defects, not the least of which is the fact that it simultaneously determines threonine for which a correction must be applied.

In view of the uncertainties concerning the alanine analyses we submitted samples of the proteins to Dr. Sidney Udenfriend, of the Department of Chemistry, New York University Medical School, who kindly undertook analyses by the isotope dilution procedure of Keston, Udenfriend, and Cannan (25). He found 8.56 per cent alanine in aldolase and 6.73 per cent in the dehydrogenase, in contrast to our values of 7.87 and 5.92 per cent respectively. Since the analyses with radioactive *p*-iodophenylsulfonyl chloride were controlled with respect to recovery from artificial amino acid mixtures and would have been low rather than high if partial racemization had occurred during hydrolysis, we accept them in preference to our own.

In addition to the analysis for alanine, Dr. Udenfriend also carried out analyses for proline and glycine. The proline results were about 9 per cent lower than the microbiological assays and, since they were performed with the DL-proline derivative as carrier, a second analysis of the dehydrogenase was made with the L-proline derivative as carrier. No significant difference was observed in the results. The discrepancy may be due in part to impurities in the standard. Although the L-proline sample employed exhibited the proper optical rotation and theoretical nitrogen content, it liberated about 0.2 per cent primary amino nitrogen in the manometric Van Slyke apparatus. The control analyses compared favorably with the previously published figures also obtained microbiologically (Tables VII

TABLE VII

Amino Acid Analyses of Crystallized Bovine Serum Albumin

Amino acid	Previous work		Present work	
	Gm. per 100 gm. protein	Method*	Gm. per 100 gm. protein	Method†
Glycine	1.96	*a*	2.0	LM
Valine	6.5	*b*	6.6	LA, LM
Leucine	13.7	"	13.2	LM
Isoleucine	2.9	"	2.7	"
Cystine	6.52	"	6.50	Chem[1]
			6.2	LM
Methionine	0.81	"	0.80	"
Serine	4.5	"	4.9‡	"
Threonine	6.5	"	7.1‡	Chem[3]
Arginine	6.2	"	5.9	LM
Histidine	3.80	"	4.00	"
Lysine	12.42	*a*	11.9	"
Phenylalanine	6.2	*b*	6.05	"
Tyrosine	5.53	*a*	5.50	Chem[5]
Tryptophan	0.58	*b*	0.58	Chem[7]
Proline	5.6	"	5.5	LM
Aspartic acid	10.25	*a*	10.45	"
Glutamic "	16.95	"	17.0	LA

* Method *a*, Shemin (28). Method *b*, Brand (26).

† For the key to the methods used, see Table I.

‡ In order to facilitate the comparison with Brand's figures we have followed his procedure here of applying an arbitrary 10 per cent correction for the destruction of serine and threonine during the hydrolysis.

and VIII). A discrepancy of this type suggests the possibility that partial destruction during hydrolysis, which is difficult to establish, will undoubtedly lead to low results in isotope dilution analysis but may yield products which are metabolically active in microbiological assay. The isotopic glycine results were aldolase 5.61 per cent and dehydrogenase 6.88 per cent, as compared with microbiological values of 6.12 and 6.98 per cent respectively.

TABLE VIII

Amino Acid Analyses of Zinc Insulin

Amino acid	Previous work* gm. per 100 gm. protein	Present work	
		Gm. per 100 gm. protein	Method†
Glycine	4.6	4.5	LM
Valine	8.8	9.1	"
Leucine	13.4	13.0	"
Isoleucine	2.9	2.8	LA
Cystine	11.6	11.7	Chem[1]
Serine	5.8	6.6‡	LM
Threonine	3.16	3.5‡	Chem[3]
Arginine	3.47	3.4	LM
Histidine	5.28	5.30	"
Lysine	2.6	2.4	"
Phenylalanine	7.9	7.95	"
Tyrosine	12.3	12.2	Chem[5]
Glutamic acid	20.2	19.9	LA
Proline	2.9	2.9	LM

* Brand ((26) p. 198).

† For the key to the methods used see Table I.

‡ See the foot-note to Table VII.

Because the glutamic acid levels are of considerable interest, we employed, in addition to *Leuconostoc mesenteroides* and *Lactobacillus arabinosus*, S. R. strain 12 of *Clostridium welchii* which Gale has shown to decarboxylate specifically L-glutamic acid in protein hydrolysates (27). Our experiments with this method have not been extensive but the results tend to confirm the previously determined figures.

During the process of standardizing our methods, we carried out analyses of crystallized bovine serum albumin, No. 46, from Armour and Company, and crystallized zinc insulin, 24 activity units per mg., from Eli Lilly and Company. Different and possibly more highly purified samples of these proteins had previously been subjected to intensive analysis by Brand and coworkers by chemical and microbiological procedures (26). Isotope dilution analyses of bovine serum albumin have been performed by Shemin (28).

The control analyses agree rather well with the previously published work (Tables VII and VIII). Early analyses for isoleucine were very much higher than Brand's figures and we were led to suspect the presence of isomeric impurities in our standard DL-isoleucine. Subsequent assays with a DL-isoleucine standard that had been shown by Dr. M. S. Dunn to be about 97 per cent pure by solubility studies indicated that our original sample contained about 19 per cent impurity, presumably the *allo* isomer. Assays with the new DL-isoleucine standard, as shown in Tables VII and VIII, are now slightly lower than the values of Brand.

The discrepancies in the serine and threonine values find no ready explanation and seem to be due in part to defects in the methods, some of which have been discussed previously. Although it has not yet been possible to evaluate all of the methods on an absolute basis, the control analyses speak well for their reproducibility in different hands.

We are indebted to Dr. M. S. Dunn and coworkers for helpful advice and for samples of glycine media and of L-leucine and DL-isoleucine of known purity.

SUMMARY

1. Aldolase and D-glyceraldehyde phosphate dehydrogenase have been subjected to complete amino acid analysis by microbiological and chemical procedures.

2. Within the limits of errors of the methods, all of the nitrogen of aldolase has been accounted for by its content of eighteen amino acids.

3. The nitrogen of D-glyceraldehyde phosphate dehydrogenase is likewise accounted for by its content of eighteen amino acids and diphosphopyridine nucleotide.

4. The minimum molecular weight of the dehydrogenase calculated from five observed residue numbers is 99,000 and that of aldolase is 140,000.

5. Analyses of crystallized bovine serum albumin and of crystallized zinc insulin are presented. These analyses for the most part are in agreement with previously published figures.

6. Included in the results are isotope dilution analyses for glycine, proline, and alanine by Dr. Sidney Udenfriend of the Department of Chemistry, New York University College of Medicine.

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THE METABOLISM OF *p*-AMINOSALICYLIC ACID IN THE ORGANISM OF THE RABBIT

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The importance of *p*-aminosalicylic acid in the treatment of tuberculosis has been demonstrated both clinically and experimentally (1–6). The close structural relationships between benzoic, salicylic, *p*-aminobenzoic (PABA), and *p*-aminosalicylic (PASA) acid warrant a comparative study of the metabolism of these substances in the animal body.[1] Recent workers, interested in the behavior of PABA in man, the dog, and the rabbit, have concerned themselves chiefly with the acetylation of this compound, with little consideration for other possible metabolic reactions such as conjugation with glycine to form the conjugate, *p*-aminohippuric acid. It is of interest to note that *in vitro* synthesis of this latter compound from PABA by homogenates and tissue liver slices has been demonstrated recently (8–10). In a comprehensive study of the fate of salicylic acid in man (11), 80 per cent of a single dose was excreted by the kidneys; of this 20 per cent was unchanged, 55 and 25 per cent in conjugation with glycine (as salicyluric acid) and glucuronic acid respectively, and a small amount (4 to 8 per cent) as gentisic acid and other products of oxidation. In children and in febrile patients, oxidation of salicylic acid was increased, while, in fever, the conjugation to form salicyluric acid was diminished. Lutwak-Mann (12) isolated gentisic acid from the urine of rats to which salicylic and acetylsalicylic acids had been fed, and observed that when the animals were poisoned with carbon tetrachloride or yellow phosphorus the formation of gentisic acid was decreased. Detailed studies of the fate of salicylic acid in the rabbit are not available. In general, however, as pointed out by Quick (7), the conjugation of glycine with an aromatic carboxyl group is markedly inhibited by a substituent group in the ortho position as in salicylic acid. Systematic studies of the metabolism of salicylic acid and related compounds with more specific methods for the determination of products of metabolism are needed. It has seemed, therefore, desirable to have information as to the biological behavior of PASA in as many animals as possible in view of the marked species variations known with other aromatic acids, *e.g.*, benzoic acid in man, the dog, or the rabbit. As an initial contribution to this problem, we present here the results of an investigation of the metabolism of PASA in the rabbit.

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[1] For a review of older work on the metabolism of substituted benzoic acids, the studies of Quick (7) should be consulted.

EXPERIMENTAL

Collection of Specimens—Male rabbits, 2 to 4 kilos in weight, were maintained on a diet of cabbage and oats. The procedures of caging and the collection of the urine were those usually employed in this laboratory (13). Decomposition was prevented by the addition of 10 ml. of 1 per cent nitric acid to the collection bottle. The PASA was administered orally through a stomach tube either as a solution of the sodium salt or as the acid in fine suspension in water, or was injected intramuscularly as a freshly prepared solution of the sodium salt.

Compounds Fed; Stability—The PASA was crystallized from absolute alcohol and dried over phosphorus pentoxide. The crystalline white material melted at 150° (decomposition) and contained 9.09 per cent of nitrogen (theoretical, 9.15 per cent) on analysis (semimicro-Kjeldahl). A 10 mg. per cent solution of the acid had an initial pH of 3.2, which changed to 4.2 on standing at room temperature for 48 hours and to 4.7 in a week; after being heated on the steam bath for 1 hour, the pH of the solution was 5.4. This change in reaction is believed to be due to a slow decarboxylation of the compound in aqueous solution with the formation of *m*-aminophenol. As shown by the iron reaction (to be discussed subsequently), the decarboxylations in 24 and 48 hours were 41 and 54 per cent respectively. When the material was dissolved in 4 per cent toluenesulfonic acid (pH 1.0), only 18 per cent decarboxylation occurred at room temperature in 24 hours, although this reaction was complete after heating the solution on the steam bath for 1 hour.

Crystallized PASA was suspended in 95 per cent alcohol and the mixture was carefully neutralized with alcoholic sodium hydroxide until a definite pink color with phenolphthalein was obtained. This solution of sodium salt was poured slowly into cold ether, with shaking. The precipitated sodium salt was filtered, washed with cold ether, and dried. The salt appeared to form a loose molecular compound with ether but the solvent could be removed by continuous evacuation at 0.5 mm. pressure over phosphorus pentoxide until the weight became constant. A sample thus dried gave a loss of 14.31 per cent. The dried sample contained 7.48 per cent of nitrogen (theoretical 7.11 per cent). In all cases in which the sodium salt was administered orally to the rabbits, the amount administered is calculated as the dried salt. The sodium salt was very soluble in water. A 100 mg. per cent aqueous solution of the sodium salt (pH approximately 6.8) appeared to be stable even at a pH of 9.0 and no decarboxylation (as was evidenced by the iron reaction) occurred when the solution was heated on the steam bath for 1 hour. There was slight discoloration in concentrated solutions on exposure to light, but samples of the dry sodium salt have been kept for several months without deterioration.

Methods of Analysis—Two color reactions have been employed to follow the metabolism of PASA. Compounds containing the orthohydroxybenzoyl group give an amethyst color with ferric salts in an acid medium, due to the formation of an iron complex. The quantitative aspect of this test has been the subject of a careful study by Nicholls (14) and Brodie and coworkers (15). In PASA, the color formation should not take place if the OH group has been blocked by substitution or if decarboxylation of the COOH group has occurred. 2-Hydroxy-4-aminohippuric acid (the PASA analogue of salicyluric acid) also gives a similar color, but of different intensity.

The free arylamino group is characterized by the lemon yellow color formed with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent). This reaction has been applied to the determination of sulfanilamide derivatives in plasma and urine (16–19). Morris (20) has shown that, for the successful application of the *p*-dimethylaminobenzaldehyde reaction to the estimation of sulfanilamides, the test must be carried out in acid media (pH 1.5 to 1.7) and in the presence of an excess of the aldehyde reagent. Both of these conditions are met in our procedures by carrying out the reaction at a pH of 2.0 to 2.2 and adding a constant amount of Ehrlich's reagent buffered with a mixture of sodium acetate and acetic acid. N-Acetylated PASA did not give this reaction and could be estimated after hydrolysis with acid as *m*-aminophenol.

Reagents—

1. 20 per cent *p*-toluenesulfonic acid (TSA). 200 gm. of TSA monohydrate (21, 22) were dissolved in 600 ml. of water, stirred with a small amount of norit K for 10 minutes, filtered, and the filtrate made up to 1 liter. 2 volumes of this stock solution were diluted with 3 volumes of water to give 8 per cent TSA.

2. Iron reagent. A 1.68 per cent solution of $Fe(NO_3)_3 \cdot 9H_2O$ (Merck) in 0.07 N nitric acid.

3. Ehrlich's reagent. 2 gm. of *p*-dimethylaminobenzaldehyde (Eastman) were dissolved in 100 ml. of glacial acetic acid and added to an equal volume of a 4 M solution of anhydrous sodium acetate. When 1 ml. of this reagent was added to 10 ml. of 4 per cent TSA, the pH was 2.0 to 2.2.

Measurements of Color—All measurements of color intensity were made with a Coleman Universal spectrophotometer (model 11). The blank was set at 100 per cent transmission and matched tubes were used for containers.

Standard K Values—A sample of PASA, twice crystallized from absolute alcohol and dried over phosphorus pentoxide, was used to prepare a standard stock solution containing 1 mg. per ml. Since PASA is not freely soluble in water and is stable in aqueous solution only as the sodium salt, the acid (100 mg.) was neutralized with 1 per cent sodium carbonate solution and made up to 100 ml. For the iron reaction, a 10-fold dilution of the stock solution was made. 1 to 8 ml. samples were added to tubes containing 2 ml. of 20 per cent TSA and the volume was made up to 10 ml., so that the final concentration of TSA was 4 per cent. 2 ml. of the iron reagent were added to each tube and the color was read at 500 mμ with a water blank similarly treated. There seemed to be no delay in the development of the color, nor any fading within a reasonable length of time. However, the readings were taken between 5 and 20 minutes. The average *K* value for the concentrations of from 0.1 to 0.8 mg. was used for the calculations of the unknowns.

For the Ehrlich reaction, the stock solution was so diluted that 1 ml. of the diluted solution contained 0.005 mg. of PASA. The procedure as outlined above was followed, except that 1 ml. of Ehrlich's reagent was used to develop the color, which was read at 450 mμ. Here also the color developed immediately and was quite stable; readings were usually taken between 5 and 20 minutes. The average *K* value obtained over the concentration range of 0.005 to 0.040 mg. was used for calculations.

Similarly the average *K* value (Ehrlich) for *m*-aminophenol was obtained for a range of 0.005 to 0.040 mg. to calculate the *m*-aminophenol (MAP) produced after hydrolysis. As would be anticipated, this value agrees very well with that obtained directly from known amounts of PASA after hydrolysis in 4 per cent TSA.

Iron Reaction—To 5 ml. of urine so diluted as to contain 0.2 to 1.2 mg. of PASA, 5 ml. of 8 per cent TSA were added, followed by 2 ml. of the iron reagent. The pink color was read at 500 mμ with a similarly treated normal urine blank for 100 per cent transmission.

Ehrlich's Reaction—(*a*) The *free* arylamino group was estimated by adding 5 ml. of 8 per cent TSA to 5 ml. of a suitably diluted urine sample, followed by 1 ml. of Ehrlich's reagent. The color was read at 450 mμ with a similarly treated normal urine blank for 100 per cent transmission. (*b*) For the determination of *total* arylamino groups, 5 ml. of the suitably diluted urine were hydrolyzed with 5 ml. of 8 per cent TSA in a loosely stoppered graduated tube in a boiling water bath for 1 hour. The tube was then cooled, the level of the fluid again brought to the mark as necessary, and the color developed with 1 ml. of Ehrlich's reagent. A similarly treated normal urine was used as the blank and the color was read at 450 mμ. Since PASA, under the above conditions, is completely decarboxylated to MAP, the results calculated as MAP were converted into PASA by multiplying by the factor 153/109. In the above estimations, the 24 hour sample of urine previous to the administration of PASA was suitably diluted to give suitable blanks to correspond to 6 and 18 hour specimens of normal urine.

Glucuronic acid was estimated by the modification of the Maughan, Evelyn, and Browne method (13) commonly used in this laboratory, and creatinine and urinary sulfur partition by the methods of Folin. PASA did not interfere with the estimations of glucuronic acid or creatinine.

DISCUSSION

150 mg. daily of PASA (as the sodium salt) per kilo of body weight could be administered orally to male rats for 2 weeks without signs of toxicity or impairment of growth. To determine whether any significant fraction of the dosage escaped absorption, the feces were analyzed for PASA in two experiments with rabbits. The amount found was so small, less than 5 mg. in a 24-hour feces, that it was thought that the routine analysis of the feces was not necessary.

In Table I, the results of urinary excretion of PASA and derivatives based on the Ehrlich reaction are summarized. 53 to 80 per cent of the compound ingested orally could be accounted for in the urine. On the other hand, when the compound was administered by intramuscular injection, there was a greater recovery, about 90 per cent, as indicated by the Ehrlich reaction. In either case, 70 to 90 per cent of the total amount of PASA recovered was excreted in the first 6 hours, and during this period 55 to 80 per cent of the amount excreted was in the acetylated form. The lower levels of acetylation, about 40 per cent in the 6 hour period, when the compound was administered parenterally, may be due to the rapidity of excretion. The rapid elimination of PASA makes it improbable that the fraction not recovered could be stored in the tissues. It has been believed that a certain amount of salicylic acid may be destroyed in the organism (23). This is in contrast to the unsubstituted compound, benzoic acid, which may be almost completely accounted for in the urine after administration.

Urine was collected from four rabbits, each fed 1 gm. of PASA on 2 successive days. The sample was treated with neutral lead acetate. The precipitate was centrifuged and the centrifugate was made just alkaline with ammonium hydroxide and treated with basic lead acetate. The precipitate was centrifuged and was decomposed by treatment with hydrogen sulfide. The lead sulfide was filtered, washed, and the filtrate and the washings, after aeration, were kept in the refrigerator for 72 hours. The crystalline white solid (400 mg.) was filtered, washed with a small quantity of ice-cold water, and dried in the desiccator. The acetyl PASA thus obtained melted at 212° with decomposition. The mother liquor was extracted four times with 200 ml. portions of ether and from the ether extract 1 gm. of an impure white solid was obtained. On washing this with small quantities of ether, 150 mg. of the sparingly soluble acetyl compound remained on the filter. The ether-soluble fraction (800 mg.) after crystallization from absolute alcohol was identified as PASA. The acetyl compound was purified by solution in dilute sodium bicarbonate solution (charcoal) and precipitation with dilute hydrochloric acid. The sample thus obtained was filtered, washed with cold water, dried first in a desiccator, and finally in an oven at 60°. The isolated compound and a pure sample of acetyl PASA were heated in a bath whose temperature was rising slowly. They melted at 212° and 214° respectively with decomposition.[2]

TABLE I

Urinary Excretion of PASA and Derivatives by Rabbits (Ehrlich Reaction)

The sodium salt used for oral administration was prepared as described in the text. For injection, the sodium salt was prepared freshly by the neutralization of the PASA with the theoretical amount of sodium bicarbonate.

PASA administered	Method of administration	PASA excreted				PASA acetylated, of total excreted	
		6 hrs.		24 hrs.		6 hrs.	24 hrs.
		Unchanged	Acetylated	Unchanged	Acetylated		
mg.		*per cent of intake*	*per cent of intake*	*per cent of intake*	*per cent of intake*	*per cent*	*per cent*
397	Salt, oral	21	42	22	58	67	72
397	" "	25	34	26	48	58	65
502	" "	16	45	18	62	74	77
362	" "	11	27	13	40	70	75
330	" "	9	39	10	53	82	84
571	Acid, "	20	25	25	47	56	65
796	" "	14	27	17	36	65	68
750	" "	12	22	17	42	65	71
580	Salt, intramuscular	46	28	48	39	38	45
740	" "	47	32	49	42	40	46

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[2] According to a private communication, Mr. Leonard Doub has observed a decomposition point of 233–234° (uncorrected) in a bath when the temperature was increased rapidly. The decomposition point was not precise and depended on the rate of heating.

A mixed sample of the two showed no depression in the melting point. Found, nitrogen 7.23 per cent; theory, nitrogen 7.19 per cent. The isolated acetyl derivative was also analyzed by the color reaction with the iron reagent to give 98.5 per cent purity and after hydrolysis with 4 per cent TSA for 1 hour under the usual conditions, as *m*-aminophenol, 98 per cent, by the Ehrlich reaction.

Since in most experiments the PASA was administered orally, an attempt was made to demonstrate whether the contents of the stomach or the intestines would decompose the compound *in vitro*. The stomach and the small intestines of rats and rabbits were removed separately and the contents were homogenized with water in a Waring blendor. PASA or its sodium salt was incubated with the emulsion for a definite period, and the mixture was then treated with a sufficient quantity of 20 per cent TSA to make a final concentration of 4 per cent TSA and filtered. The filtrate, centrifuged as necessary, was analyzed by the iron and the Ehrlich reactions. The results were also checked by estimating by the Ehrlich reaction the *m*-aminophenol formed after heating the filtrate for 1 hour on the steam bath. All necessary dilutions were made in 4 per cent TSA. A control experiment was run in each case without PASA, which served as a blank in the estimations. The results are summarized in Table II.

TABLE II

Incubation of PASA with Homogenized Gastric and Intestinal Contents of Rabbit and Rat

In all experiments except Experiment 19-9, the contents were incubated with finely divided PASA; in Experiment 19-9 the acid was dissolved in 4 ml. of 5 per cent sodium bicarbonate.

Experiment No.	Organ	Time of incubation	PASA	PASA recovered			
				Iron reaction	Ehrlich		
					Free (*a*)	Total (*b*)	Conjugated (*b* − *a*)
		min.	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*
19-9 (rabbit)........	Stomach	90	104	97	84	98	14
	Intestine	210	101	103	80	98	18
20-10 (rabbit).......	Stomach	90	108	96	70	92	22
	Intestine	210	101	97	79	96	17
21-11 (rat)..........	Stomach	90	32	94	81	91	10
	Intestine	210	50	92	86	94	8

One might have expected the decarboxylation of PASA, associated with the acidity available in the stomach and the activity of the intestinal flora.

However, the results of the incubation of PASA with gastric and intestinal contents of the rabbit and the rat definitely indicated that there was no decarboxylation *in vitro*, since essentially all the material originally added was recovered by the iron reaction. The only change detected was a small amount of acetylation, 14 to 23 per cent in the case of the rabbit and 6 to 10 per cent with the rat. This needs further investigation.

The earlier experiments indicated conjugation with acetic acid to give the N-acetyl derivative. However, PASA might be conjugated in the animal body in more than one way, as there are three groups in the molecule which might be used in such reactions. Owing to the ease with which the compound is decarboxylated in the test-tube, it might have been expected that this reaction would take place in the animal body. If this were the case to any considerable extent, the resulting *m*-aminophenol should be acetylated or possibly conjugated with glucuronic acid or sulfuric acid (24). One might also observe the introduction of a hydroxyl group in the molecule of PASA *in vivo*, either by direct oxidation of the compound and its acetyl derivative or by a formation and rearrangement of the hydroxyamino derivative,[3] to form 2,5-dihydroxy-4-amino- or acetylaminobenzoic acid. These compounds might be conjugated with glucuronic or sulfuric acid, particularly at the hydroxyl group in the 5 position.

Studies of urinary sulfur after feeding PASA indicated practically no increase in ethereal sulfate. No uniformity is displayed in the excretion of extra glucuronic acid after the administration of PASA. From experience in this laboratory, we consider that, with rabbits, any value for extra glucuronic acid less than 70 mg. either in the 6 hour or 18 hour period is of little significance. In only two of ten experiments was the extra glucuronic acid in the 6 hour period, the period of rapid excretion of PASA, greater than 70 mg., values of 75 and 103 mg. being obtained. On this basis we are inclined to believe that the amounts of extra glucuronic acid after the administration of PASA orally or parenterally are not indicative of any significant conjugation.

It may, therefore, be assumed that there is neither considerable decarboxylation of PASA nor introduction of a new hydroxyl group *in vivo*, and the amounts of free and combined derivatives in the urine as estimated by the Ehrlich reaction represent primarily free and acetylated PASA.

The question of the conjugation of glycine with the carboxyl group of PASA may be discussed. 2-Hydroxy-4-aminohippuric acid gives a color with the iron reagent similar to those with PASA and acetyl PASA; however, it is not decomposed completely to *m*-aminophenol under the conditions used for hydrolysis, but still gives considerable color with the iron reagent after such a procedure. The experimental urine did not give any color with the iron reagent after hydrolysis, indicating the absence of the glycine conjugate. This is in accordance with the belief that there is little or no conjugation of glycine with ortho substituted benzoic acids with the exception of *o*-chlorobenzoic acid (7). It may be noted, however, that a considerable proportion of salicylic acid, an orthohydroxyl derivative of benzoic acid, when fed to man, is reported to be excreted in conjugation with glycine (11).

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[3] Williams (25) has discussed the possible formation of *p*-hydroxylaminobenzenesulfonamide in metabolism. A number of hydroxy derivatives of the sulfonamides have been isolated and characterized in studies both *in vivo* and *in vitro* (25–27).

The color observed in the iron reaction with the experimental urine should be due to the presence of both the free and acetylated PASA. It may be pointed out that, since the two compounds give different intensities of color with the iron reagent, the two K values being 0.524 and 0.264 respectively under our experimental conditions, the evaluation of the observed optical activity in terms of PASA alone might suggest considerable decarboxylation of the compound *in vivo*. Therefore, the values for PASA and acetylated PASA obtained by the Ehrlich reaction with the experimental urines have been used to calculate the expected individual optical densities in the iron reaction. The difference between the *observed* optical density and the sum of the *calculated* optical densities of the two forms of PASA present might well be due to the presence of small amounts of other metabolites.

The presence of *m*-aminophenol and its N-acetyl derivative, which do not react with the iron reagent, would give negative values in such a calculation, while the presence of β-resorcylic acid, which reacts with the iron reagent, would give positive values. The difference ("unknown") has been somewhat arbitrarily expressed as PASA for the calculation of the net results of the iron reaction (Column 3). The values calculated in this manner are presented in Table III.

It may be noted that the amounts of unknown metabolite thus calculated as differences represent a relatively small proportion of the total PASA (free and acetylated) excreted and that the values are both positive and negative. The data fail to offer any significant evidence of decarboxylation of PASA in the organism of the rabbit. This would be shown by consistent negative values. On the contrary, the differences, thus calculated for the 6 hour period, the period in which maximal excretion of the PASA is occurring, are only slightly positive in six of the ten experiments recorded and none of the negative values represents any large proportion of the total amount excreted.

The algebraic sum of the amounts in Columns 1, 2, and 3 represents the total amount of PASA or derivatives excreted, as indicated by the iron reaction. The per cent of the total PASA administered which is represented by this value is calculated in Column 4. This figure may be compared with the total PASA, excreted in the 6 hour period as obtained by the Ehrlich reaction, which is shown in Column 5. The average total recoveries for the eight feeding experiments as calculated by the two methods are 45.3 and 48.6 per cent respectively. The differences between these average values are not significant.

We are indebted to Mr. Leonard Doub of Parke, Davis and Company for generous gifts of *p*-aminosalicylic acid and some of its derivatives and for the analysis of the sodium salt of PASA, and to Dr. A. C. Bratton, Jr., for suggestions relating to the colorimetric methods of analysis used in this study. In particular the interest and helpful criticism of Dr. Bratton are gratefully acknowledged.

TABLE III

Interpretation of Iron Reaction in Terms of Excretions of PASA and Derivatives As Determined by Ehrlich Reaction

The calculations are for the 6 hour period immediately after the administration of PASA. The "unknown" metabolites (Column 3) are expressed in terms of PASA. Column 5 is included for comparison. For explanation of the calculation, the text should be consulted. The PASA administered can be obtained from the corresponding data of Table I.

Free (1)	Acetyl (2)	Unknown (3)	Sum of intake (4)	Ehrlich reaction of intake (5)
mg.	*mg.*	*mg.*	*per cent*	*per cent*
82	165	−42	52	63
98	136	−27	52	59
82	228	−34	55	61
41	98	+9	41	38
29	129	−29	39	48
112	145	+13	47	45
113	214	+5	42	41
88	162	+4	34	34
268	165	+17	78	74
351	239	+87	91	79

SUMMARY

1. An aqueous solution of *p*-aminosalicylic acid (PASA) undergoes a gradual decarboxylation at room temperature with the formation of *m*-aminophenol. This is evidenced by the change in pH toward the alkaline side as well as by the decrease in the intensity of the reaction with ferric salts. The sodium salt, however, forms a stable solution when dissolved in water.

2. The metabolic fate of PASA in the rabbit has been studied. PASA

is rapidly excreted in the urine in the unchanged and acetylated forms. From 40 to 60 per cent of the amount administered orally could be accounted for in 24 hours as the acetyl derivative. Both free and acetylated PASA have been isolated from the experimental urines.

3. PASA is absorbed almost completely from the gastrointestinal tract and there is no indication of decarboxylation *in vitro* in the presence of gastric or intestinal contents. No clear-cut evidence of any appreciable decarboxylation *in vivo* has been obtained.

4. Data obtained for extra glucuronic acid do not suggest any significant conjugation as a glucuronide, while the possibility of conjugation with glycine appears remote. No increase in ethereal sulfate formation is indicated.

5. The chief mechanism of detoxication of PASA in the rabbit appears to be through acetylation.

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PASSAGE OF SELENIUM THROUGH THE MAMMARY GLANDS OF THE WHITE RAT AND THE DISTRIBUTION OF SELENIUM IN THE MILK PROTEINS AFTER SUBCUTANEOUS INJECTION OF SODIUM SELENATE

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One of the pathways by which selenium may be eliminated from the animal body other than through the kidney, gastrointestinal tract, and lung is through the mammary glands into the milk of lactating animals. This has been shown by several investigators to take place in certain domestic animals. Dudley (1) found 0.02 to 3.00 parts per million of selenium in the milk of selenized cows. Smith *et al.* (2) reported in an extensive survey that milk from cows grazing in seleniferous areas contains 16 to 127 γ of selenium per 100 cc. Moxon (3) observed that selenium was carried in the milk of selenized animals to such an extent that nursing calves often showed all the prominent symptoms of "alkali" disease.

Organoselenium as obtained from seleniferous grains (Franke and Potter (4)) and inorganic selenium, when administered to experimental animals, have been shown to pass into the proteins of liver[1] (Westfall and Smith (5)), muscle,[1] the plasma fractions[1] (Westfall and Smith (5)), and hemoglobin.[1] In view of the fact that there is a certain fixation of selenium in tissue proteins, the question may be raised whether selenium of the milk exists in the milk proteins and, if so, in what protein fractions. In order to obtain information about the nature of selenium in the milk of selenium-injected white rats, various experiments were carried out.

The experiments reported here were carried out with the radioactive isotope of selenium. With the use of radioselenium which has the advantages of a half life of 50 days with a specific γ-ray emission of 0.05 and 0.21 m.e.v., microdeterminations of selenium were made with far greater accuracy than are possible by any known chemical analytical procedure. The extreme sensitivity of the method made possible quantitative detections of traces of selenium.

EXPERIMENTAL

It was first established, as it has been in domestic animals, that selenium passes through the mammary glands of the white rat. This was accom-

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[1] Unpublished data of the author.

plished in the following manner: Five lactating rats, having three to six pups, 2 to 3 days old, were injected either with single or multiple, subcutaneous subtoxic amounts of sodium selenate containing radioselenium. 24 hours after the last parental injection, the pups were sacrificed by decapitation and wet ashed separately; radioactivity of the ash was determined on a dipping Geiger-Müller counter of a scale-of-four.

Studies on the distribution of selenium in the milk necessitated the fractionation of the various milk proteins. The milk for these distribution studies was collected as follows: Two lactating white rats having six and nine pups respectively, 10 to 14 days of age, were injected with sodium selenate containing radioselenium. The pups were separated from their mothers for 12 to 18 hours, after which they were returned and allowed to suckle for 1 to 3 hours. The pups were then sacrificed, the stomachs excised, and the stomach contents, which consisted of milk curd, removed. The milk of the two litters was pooled separately and the milk samples were agitated with a small amount of benzoic acid to break up the curd (Mayer (6)).

Total proteins of the milk obtained from Litter 1 were precipitated with trichloroacetic acid. The milk from Litter 2 was divided into three aliquots and three protein fractions were obtained with the method of Moir (7): (*a*) *Casein* was obtained by isoelectric precipitation at pH 4.6; (*b*) *casein and globulin* fractionation was obtained by saturating the milk solution with magnesium sulfate at 20°; and (*c*) *total protein* was precipitated by heating with 4 per cent trichloroacetic acid.

On each of the above protein fractionations radioactive determinations were made on the filtrate and precipitate. All the precipitates were washed twice.

The globulin content of the milk was obtained by subtracting the casein fraction from the casein plus globulin fraction. Albumin was determined by the analysis of the filtrate from the casein plus globulin precipitation. In this manner it was possible to estimate the distribution of selenium in total proteins, albumin, globulin, and casein fractions.

RESULTS AND DISCUSSION

The results from these two series of experiments will be considered under separate headings; *i.e.*, evidence that selenium passes through the mammary glands of the white rat, and distribution of selenium in the various milk proteins.

Presence of Selenium in Milk of White Rat—The results for this section are shown in Table I, where the concentration of radioactive selenium in the pups is expressed both as per cent dose per pup and per cent dose per litter. Upon inspection of Table I it will be noted that after both single and multiple subcutaneous injections of sodium selenate 0.33 to 2.3 per cent of the dose per pup or 2.0 to 9.3 per cent of dose per litter was transferred through the milk of the lactating mother to the suckling young. It must be borne in mind that the actual amount of selenium transferred through the mammary glands of the mother to the young during the experimental period outlined here is not truly represented in the values expressed as per cent dose per litter. These values represent a fraction of the actual amount of selenium secreted; the exact amount transferred through the glands is undoubtedly greater because part of the selenium received by the young via milk is excreted through the kidney, gastrointestinal tract, and lungs. The values expressed as per cent of the dose per litter are representative of the amount of selenium in the litters 24 hours after the last parental injection. These data, however, are sufficient to show that selenium, when administered to lactating mothers as an inorganic salt, is transferred through the mammary glands of the white rat and that at least 2.5 to 9.3 per cent of the dose is present in the suckling young 24 hours after the last parental injection.

TABLE I

Passage of Selenium (Radio) through Mammary Glands of White Rat after Subcutaneous Injection of Sodium Selenate (Na_2SeO_4)

Experiment	No. of parental injections	Total selenium injected	Total count injected	No. of pups suckled	Per cent dose administered per pup		Per cent dose administered per litter
					Average	Range	
		mg.	*per min.*				
A	4	1.12	324	4	2.30	1.8 -3.4	9.3
B	3	0.62	544	3	1.60	1.4 -2.0	5.0
C	3	0.76	3960	6	0.42	0.36–0.50	2.5
D	1	0.58	1555	6	1.61	0.36–0.71	3.7
E	1	0.58	1555	6	0.33	0.20–0.43	2.0

The list of animals that may secrete selenium through the mammary glands is, therefore, extended to include the white rat.

The mammary gland of one lactating animal was found to contain 0.058 per cent of the dose per gm. of tissue 24 hours after the last injection. This value corresponds to about the value found for per cent of the dose per gm. of pup and about a fourth the value for liver (0.208) in the same time interval.

Distribution of Selenium in Various Milk Proteins—The results obtained for this section are given in Table II. Perhaps the most outstanding finding here is that 91 per cent of the selenium present in the milk obtained from Rat 1 was in the protein fraction, while in Rat 2 *all* the selenium was present in protein fractions.

Of importance here is the fact that *inorganic selenium* is converted to some *organoselenium protein complex.* This conversion of inorganic selenium to organoselenium has been shown to occur in other tissue proteins; *i.e.*, the liver, muscle, hemoglobin, and plasma fractions. The value and significance of these findings in relationship to the metabolism of selenium and the closely related sister substance, sulfur, cannot be stated but must await further elucidation and experimentation. Smythe and Halliday (8) were able to demonstrate in *in vitro* studies that inorganic sulfur (radiosulfur) as sodium sulfide may be converted in the presence of a suitable liver enzyme to cysteine sulfur. This established without a doubt that inorganic sulfur was converted to organic sulfur. Recently, Dziewiatkowski (9) has demonstrated that sulfide sulfur containing radioactive sulfur (S^{35}) was utilized by the intact rat for the synthesis of cystine. The radioactive sulfur from sulfide was found to be present in the cystine isolated from hair, liver, skeletal muscle, and skin.

TABLE II

Presence of Selenium (Radio) in Rat Milk Proteins after Subcutaneous Injections of Sodium Selenate (Na_2SeO_4)

	Rat 1	Rat 2
Volume of milk, cc.	0.5	2.0
Counts per cc.	26.0	46.1
Dose per cc., %	0.515	0.913
Counts in protein fraction	11.3	64.8
" " non-protein fraction	1.0	0.0
Counts recovered, %	94.5	72.0
" in casein, %		53.8 (Corrected)
" " albumin and globulin, %		46.4 "
" " casein " " %		60.8 "
" " albumin, %		39.5 "
" " globulin, %		6.9 "

Rats 1 and 2 were injected with 5050 counts of radioselenium as sodium selenate.

It may be that a similar mechanism may take place in the experiments described here in the conversion of inorganic selenium to organoselenium.

The protein fractionation experiments carried out on the milk obtained from Rat 2 show that approximately 54 per cent of the selenium present in the milk is in the casein fraction, while smaller amounts, namely 40 and 7 per cent, are in the albumin and globulin fractions, respectively. It is of interest here to point out that the presence of selenium in milk protein is not confined to any one particular protein fraction but may be detected in all the fractions, as was observed in the plasma experiments.[1] The distribution of selenium in the various protein fractions of milk is more or less proportional to the size of the fraction. Casein, which is the largest protein fraction of milk (Cox and Mueller (10)) concentrates the most selenium, while lesser amounts of selenium are present in the smaller milk protein fractions, albumin and globulin.

SUMMARY

It has been demonstrated that after subtoxic subcutaneous injections of selenium (radioactive) as sodium selenate, selenium passes through the mammary gland into the milk of the white rat. Further, it has been found that administered inorganic selenium was converted into organoselenium in the protein fraction of milk because approximately 54 per cent of the selenium present in the milk was in the casein fraction, while smaller amounts, namely 40 and 7 per cent, were in the albumin and globulin fractions, respectively.

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THE METABOLIC INTERRELATIONSHIP BETWEEN TRYPTOPHAN, PYRIDOXINE, AND NICOTINIC ACID; FORCED FEEDING STUDIES IN RATS*

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PLATE 1

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The addition of corn products to a synthetic low protein diet very low in nicotinic acid has been reported by Krehl and coworkers (1, 2) to depress growth in rats. This growth retardation could be prevented by tryptophan as well as by nicotinic acid. These findings have been confirmed in this laboratory by the paired feeding technique (3) and by other investigators (4, 5). The interchangeability of nicotinic acid and tryptophan is not peculiar to corn-supplemented rations, since a similar relationship has been demonstrated with non-corn rations which are low in both tryptophan and nicotinic acid (6, 7). The Wisconsin group has indicated that intestinal microorganisms may play a significant part in the deficiency syndrome, since the growth-depressing effect of corn can be greatly modified by the use of carbohydrates previously shown to favor intestinal synthesis (7).

An increased excretion of nicotinic acid and its derivatives has been shown to result from the administration of tryptophan (4, 5, 8, 9), which has been interpreted to indicate that tryptophan may function as a metabolic precursor of nicotinic acid. The studies which have been reported on the increased excretion of nicotinic acid following tryptophan feeding have been made with adult rats on basal diets which contained tryptophan. It was thought desirable to compare the excretion of nicotinic acid of growing rats on an acid-hydrolyzed casein diet containing a minimum of tryptophan (0.24 mg. per gm. of diet) with that obtained with an adequate amount of tryptophan (20 mg. per rat per day) and to determine whether there is any relationship between the excretion of nicotinic acid and the gain in weight.

It has been shown that in pyridoxine-deficient rats tryptophan is not normally metabolized, resulting in the excretion of xanthurenic acid in the urine (10–13). It was of interest, therefore, to study the effect of a pyridoxine deficiency on the excretion of nicotinic acid and its methylated derivative, N^1-methylnicotinamide, associated with the feeding of tryptophan.[1]

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In the investigation reported here, the metabolic interrelationship between tryptophan, pyridoxine, and nicotinic acid was studied with an acid-hydrolyzed casein diet deficient in tryptophan as well as pyridoxine and nicotinic acid.

EXPERIMENTAL

Basal Ration—The composition of the basal ration is shown in Table I. "Vitamin-free" casein (Labco) was extracted three times with hot 95 per cent ethanol and then completely hydrolyzed[2] with sulfuric acid by the method of Berg and Rose (16). The hydrolysate was first put through a spray drier[3] which reduced the moisture content to about 5 per cent, and then was completely dried in a vacuum oven at about 70°. 2 per cent of unhydrolyzed "vitamin-free" casein was added to supply strepogenin (17), which is destroyed by acid hydrolysis. All the vitamins, with the exception of nicotinic acid and pyridoxine, were provided in excess of the requirement. The very small amounts of tryptophan and nicotinic acid supplied by the basal ration are shown in Table I. The amount of pyridoxine present in the basal ration was less than could be measured accurately.

Feeding Procedure—Originally, weanling rats (Sprague-Dawley strain) were fed variable amounts of ground Purina dog checkers until the weight of each animal was within 2 gm. of 60 gm. They were then separated into ten groups of six each, with uniform distribution as to sex and litter. Thus, eight groups provided all possible combinations of the three supplements tryptophan, nicotinic acid, and pyridoxine. In addition, there were two groups receiving 2 per cent succinylsulfathiazole, tryptophan, and pyridoxine, one group without and the other with nicotinic acid.

All the animals, regardless of the supplement, ate only 3 to 5 gm. of the diet and consequently lost weight. The addition of large amounts of thiamine and of sodium glutamate did not increase the food consumption. After 2 weeks a regimen of forced feeding was started. Most of the rats receiving no tryptophan died the 1st day of forced feeding, and the remainder died within a couple of days. The animals receiving tryptophan survived 1 week of forced feeding, but they were so emaciated from the first 2 weeks of poor food consumption that it was deemed advisable to start an entire series of new animals.

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[1] After this study was started two laboratories published results of experiments on the effect of vitamin B_6 deficiency on the conversion of tryptophan to nicotinic acid and N^1-methylnicotinamide (14, 15). Again, however, the basal rations contained an adequate amount of tryptophan for growth.

[2] Grateful acknowledgment is made to Dr. M. Womack for aid in the preparation of the acid-hydrolyzed casein, and to Dr. W. C. Rose for the use of equipment in the Department of Chemistry.

[3] This was done in the Division of Dairy Manufactures by Mr. V. L. Swearingen.

The weights of 60 young rats[4] were adjusted to within 2 gm. of 80 gm. by feeding variable amounts of ground Purina dog checkers. The rats were then separated into ten groups of six each as in the first experiment.

TABLE I

Composition of Basal Ration

	per cent
Acid-hydrolyzed casein (Labco, extracted 3 times with 95% ethanol)	20.0
Labco casein	2.0
Salts 446 (Illinois)*	5.0
Corn oil	3.0
DL-Methionine	0.6
Sucrose	69.4
2 % sulfasuxidine added at expense of sucrose	
Level of incorporated vitamins	
	mg. per 100 gm.
Thiamine	0.25
Riboflavin	0.50
Calcium pantothenate	2.0
Choline chloride	100.0
Inositol	10.0
p-Aminobenzoic acid	50.0
Biotin	0.01
Folic acid	0.025
2-Methylnaphthoquinone	0.1
Halibut liver oil (diluted 1:2 with corn oil) at level of 2 drops per wk., with α-tocopherol included at 0.5 mg. per drop	
Basal ration supplied per gm.	
Tryptophan	0.24 mg.
Nicotinic acid	0.042 γ

* The composition of Salts 446, in gm., is as follows: NaCl 243.198, $K_3C_6H_5O_7 \cdot H_2O$ 533.0, K_2HPO_4 174.0, $CaHPO_4 \cdot 2H_2O$ 800.0, $CaCO_3$ 368.0, $MgCO_3$ 92.0, $FeC_6H_5O_7 \cdot 3H_2O$ 36.0, $CuSO_4 \cdot 5H_2O$ 0.4, $MnSO_4$ 2.8, $K_2Al_2(SO_4)_4 \cdot 24H_2O$ 0.2, KI 0.1, $CoCl_2 \cdot 6H_2O$ 0.2, $ZnCO_3$ 0.1, NaF 0.002, total 2250.0.

The basal ration was well mixed with an equal amount of distilled water. The desired amount of this slurry was taken up into a graduated 10 cc. hypodermic syringe to which was attached an ordinary hypodermic needle, the tip of which was blunted with a file and coated with a protective knob

[4] Obtained from the Department of Chemistry.

of solder (18). After safely inserting this needle into the rat's stomach, the measured amount of diet was gently injected. As a check on the actual amount fed, the rats were weighed immediately before and after feeding. The rats were fed three times daily, at about 5 hour intervals, with the supplement included in the first feeding. The amount of basal ration, supplement, and the average weekly gain in weight of each group are shown in Table II.

TABLE II

Average Weekly Gain in Weight in Gm. (with Standard Errors)

Wk.	Tryptophan				No tryptophan				2 per cent sulfasuxidine	
	Nicotinic acid		No nicotinic acid		Nicotinic acid		No nicotinic acid		Tryptophan + pyridoxine	
	Pyridoxine	No pyridoxine	Pyridoxine	No pyridoxine	Pyridoxine	No pyridoxine	Pyridoxine	No pyridoxine	No nicotinic acid	Nicotinic acid
1st	11.8 ±1.4	10.8 ±1.3	9.9 ±1.0	10.4 ±1.2	10.7 ±2.0	8.6 ±0.4	10.6 ±1.0	9.6 ±2.2	14.1 ±1.0	*15.9 ±1.0
2nd	15.4 ±1.7	15.7 ±1.0	13.9 ±1.2	15.4 ±1.5					16.5 ±1.9	15.9 ±1.3
3rd	16.8 ±1.1	16.3 ±2.1	17.2 ±1.0	17.8 ±0.9					17.7 ±2.1	18.7 ±1.2
Total.........	43.5 ±3.7	43.3 ±4.2	41.2 ±2.9	43.6 ±3.1					48.1 ±3.4	†50.5 ±1.9

Amount of ration given		Daily supplement		Probability of chance outcome
wk.	*gm.*		*mg.*	
1st, 2nd	7.5	L-Tryptophan	20.0	*<0.001
3rd	9.75	Nicotinic acid	0.2	†0.0077
		Pyridoxine	0.2	

Gain in Weight—Statistical examination[5] of the data by the method of analysis of variance (19) indicated no significant effect of any of the supplements upon gain in weight. However, porphyrin-caked whiskers and acrodynia of the mouth, paws, and tail were evident in the pyridoxine-deficient rats on the 13th day and all of the tryptophan-deficient rats were dead by this time.

The presence of 2 per cent succinylsulfathiazole in the diet resulted in a significantly greater gain in body weight during the 1st week, but not in subsequent weeks. Thus, there was a greater slope (Text-fig. 1) for the growth curve of the succinylsulfathiazole-fed rats during the 1st week, but

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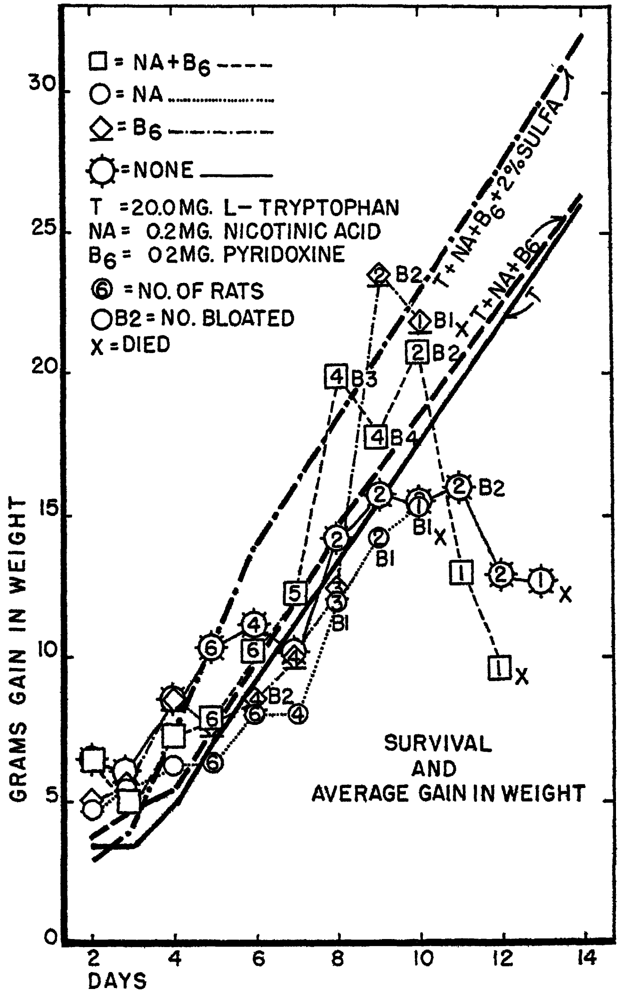
[5] Grateful acknowledgment is made to Dr. K. M. Blaxter, Commonwealth Fellow on leave of absence from the Ministry of Agriculture and Fisheries, Great Britain, for advice and instruction in the statistical treatment of the data.



TEXT-FIG. 1

thereafter the slope was about the same as for the other groups. Elvehjem and his group at Wisconsin (20) have observed a similar beneficial effect of sulfasuxidine on the weight of chicks when folic acid was included in the diet.

Trypotophan Deficiency—The tryptophan-deficient animals assumed a hunch-backed position and were unthrifty looking, with the mouth, paws, and abdominal area denuded. They progressively manifested the following symptoms: bloat, diarrhea, convulsions with screeching, and finally death. After forced feeding all tryptophan-deficient animals displayed the following peculiar behavior: (1) pawing the face as though trying to with-

TABLE III—*Average Urinary Exc*

The results are expressed as micrograms per rat per day.

		Tryptophan			
		Nicotinic acid		No nicotinic acid	
		Pyridoxine	No pyridoxine	Pyridoxine	No pyri
1st wk.	Tryptophan	152.0 ± 38.2	126.6 ± 50.3	244.6 ± 36.1	159.8 ±
	Excretion, %	0.70	0.58	1.12	0.73
	Nicotinic acid	23.0 ± 1.8	20.8 ± 1.4	14.4 ± 0.2	13.5 ±
	Conversion or recovery, %	4.30	3.65	0.066	0.062
	N^1-Methylnicotinamide	139.5 ± 18.9	76.5 ± 17.8	29.1 ± 0.6	25.0 ±
	Conversion or recovery, %	55.20	25.75	0.133	0.115
	Xanthurenic acid	0	220.0 ± 111.4	0	0
	Conversion, %	0	1.01	0	0
2nd wk.	Tryptophan	163.8 ± 28.5	158.8 ± 43.2	189.4 ± 46.6	120.4 ±
	Excretion, %	0.73	0.71	0.85	0.54
	Nicotinic acid	24.8 ± 3.4	22.9 ± 1.1	14.9 ± 0.4	14.7 ±
	Conversion or recovery, %	4.95	4.10	0.067	0.066
	N^1-Methylnicotinamide	140.2 ± 19.1	64.7 ± 11.3	27.2 ± 1.3	31.3 ±
	Conversion or recovery, %	56.5	16.7	0.122	0.140
	Xanthurenic acid	0	420.0 ± 177.2	0	216.6 ±
	Conversion, %	0	1.88	0	0.97
3rd wk.*	Tryptophan	2760.0 ± 823.8	434.5 ± 34.5	1285.6 ± 277.8	2090.0 ±
	Excretion, %	2.70	0.42	1.26	2.04
	Nicotinic acid	39.4 ± 5.4	26.7 ± 5.4	22.4 ± 0.6	19.9 ±
	Conversion or recovery, %	8.50	3.40	0.022	0.019
	N^1-Methylnicotinamide	525.6 ± 78.4	443.5 ± 125.5	129.6 ± 14.6	78.8 ±
	Conversion or recovery, %	198.00	182.35	0.127	0.077
	Xanthurenic acid	0	6083.5 ± 1416.5	0	2833.3 ±
	Conversion, %	0	5.95	0	2.77

* Urine was collected for 24 hours following a dose of 100 mg. of L-tryptophan.

draw the food; (2) crawling around the edge of the cage with the side of the face resting on the floor of the cage; (3) rearing up on the hind legs and pawing frantically with the front paws; (4) after 5 to 10 minutes they would go to sleep, with a tendency to sleep on the side.

etion (with Standard Errors)

	No tryptophan				2 per cent sulfasuxidine	
	Nicotinic acid		No nicotinic acid		Tryptophan + pyridoxine	
ioxine	Pyridoxine	No pyridoxine	Pyridoxine	No pyridoxine	No nicotinic acid	Nicotinic acid
31.1	39.4 ± 22.1 2.19	18.5 ± 9.5 1.03	22.0 ± 2.1 1.22	33.5 ± 9.5 1.86	187.6 ± 48.9 0.86	140.0 ± 29.5 0.64
0.9	13.8 ± 3.2 3.30	10.6 ± 0.1 1.40	7.2 ± 3.0 0.400	7.8 ± 1.2 0.433	13.8 ± 1.2 0.063	16.2 ± 0.7 1.20
5.2	43.0 ± 26.6 10.50	28.0 ± 8.0 5.60	22.0 ± 3.1 1.222	16.8 ± 1.8 0.933	26.2 ± 2.6 0.120	121.2 ± 13.8 47.50
	0 0	0 0	0 0	0 0	0 0	0 0
36.3					136.2 ± 15.9 0.61	125.2 ± 12.0 0.56
0.4					16.4 ± 0.7 0.074	16.0 ± 2.0 0
6.5					32.0 ± 2.4 0.143	123.6 ± 6.3 45.80
164.1					0 0	0 0
932.3					2044.6 ± 894.4 2.0	2187.0 ± 1047.1 2.14
2.1					27.0 ± 2.3 0.026	53.1 ± 6.1 13.05
30.0					191.2 ± 42.2 0.187	553.2 ± 71.7 181.00
793.5					0 0	0 0

The extent of bloat may be seen in the group receiving pyridoxine, which showed an average gain of 11 gm. per rat from the 8th to the 9th day. The first fatality occurred on the 6th day, and on the 7th day there was at least one dead in all four groups receiving no tryptophan. All the animals from the two groups receiving either nicotinic acid or pyridoxine were dead on the 10th day. The animal which lived the longest, 13 days, was from the group receiving the basal ration without any supplement.

In a supplementary experiment to determine whether tryptophan could cure as well as prevent the syndrome, twelve young rats (weighing 75 to 88 gm.) were forcibly fed the tryptophan-deficient basal ration plus nicotinic acid and pyridoxine. One animal died on the 2nd day and five more had died by the morning of the 3rd day. The animals had an unkempt appearance, bloat, diarrhea, and fits. A supplement of 40 mg. of tryptophan was then given to the remaining animals: one died the same day, but in the others the daily supplement of tryptophan eliminated the deficiency symptoms.

On postmortem examination the stomach and intestines of the tryptophan-deficient animals were found to be greatly distended and filled with liquid and gas. The liver was light colored. One rat from the group receiving nicotinic acid and pyridoxine was sacrificed for histological study on the 10th day of experiment.[6]

The cells (Fig. 1) show fatty degeneration in the cytoplasm. They are swollen, rounded, and highly vacuolated, resulting in a tenuous cytoplasm resembling chicken-coop wire. Another interesting feature is the appearance of the nuclei, which contain large masses of chromatin and have an enlarged nucleolus.[7]

Excretion Studies; Methods—Individual urinary collections were made for 24 hours in metabolism cages during the 2nd and 3rd weeks and after an oral dose of 100 mg. of L-tryptophan during the 4th week. To neutralize a possible effect of time, urine was collected from an equal number of rats from each group on any particular day. The bladder was emptied at the beginning and end of the collection period by applying pressure. The urine was analyzed for apparent free tryptophan (21) and for nicotinic acid (22), with *Lactobacillus arabinosus* as the test organism. The N^1-methylnicotinamide was determined by the acetone-fluorometric method of Huff and Perlzweig (23), and xanthurenic acid[8] by the colorimetric method of Rosen *et al.* (14).[9]

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[6] Grateful acknowledgment is made to Dr. A. V. Nalbandov, Dr. F. B. Adamstone, and Dr. C. C. Morrill for the preparation and interpretation of the liver sections.

[7] A more complete study of the histologic changes in tryptophan deficiency produced by forced feeding is planned.

[8] The xanthurenic acid was kindly furnished by Dr. J. W. Huff of Sharp and Dohme, Inc.

[9] I wish to thank Lucille D. van Ghyl, Barbara Chase, and Shirley Spaeth for assistance in some of these determinations.

Results—Average urinary excretions, expressed as micrograms per rat per day, are shown in Table III. The values given are the group means with their standard errors. The amount of nicotinic acid excreted when no nicotinic acid was added was calculated in terms of a percentage conversion from tryptophan on a weight basis. This amount was then subtracted from the nicotinic acid excretion for the corresponding group receiving nicotinic acid, and the per cent recovery of added nicotinic acid was calculated. The same calculations were made for N^1-methylnicotinamide. In Table IV the effect of each supplement, both in the presence and absence of the other supplements, is computed, and the difference is expressed as a percentage of the amount excreted when the particular supplement was not given. The mean differences were analyzed statistically by the t test (19).

Effect of Tryptophan—It is interesting that the addition of tryptophan resulted in the greatest increase in tryptophan excretion when nicotinic acid was not added. Although the general result of adding nicotinic acid was a lesser excretion of tryptophan, the differences are not statistically significant, and there are even some cases of an increased excretion.

The addition of 20 mg. of L-tryptophan increased the excretion of nicotinic acid from 78 to 86 per cent, regardless of the presence or absence of nicotinic acid or pyridoxine. The increase in N^1-methylnicotinamide due to the addition of tryptophan was considerably greater when nicotinic acid was present (204 per cent) than when it was lacking (39 per cent).

Effect of Pyridoxine—There was no significant effect of pyridoxine on tryptophan excretion in the absence of added tryptophan and at the 20 mg. level, although the trend was toward a higher excretion. When 100 mg. of L-tryptophan were fed, however, pyridoxine greatly increased the excretion of tryptophan in the presence of nicotinic acid, but when nicotinic acid was lacking, pyridoxine decreased the excretion of tryptophan. The latter was not statistically significant ($P = 0.202$).

Pyridoxine had no significant effect either on the percentage conversion of tryptophan to nicotinic acid or of tryptophan to N^1-methylnicotinamide. However, it is significant that there was a greater excretion of added nicotinic acid in the form of N^1-methylnicotinamide when pyridoxine was present. When 100 mg. of L-tryptophan were given, however, this difference was not statistically significant ($P = 0.300$), probably because of the variability in excretion of N^1-methylnicotinamide that was obtained.

Effect of Nicotinic Acid—It is of great significance that when 100 mg. of L-tryptophan were fed, 181 to 198 per cent of added nicotinic acid was recovered as N^1-methylnicotinamide, even after the amount synthesized and excreted in the absence of nicotinic acid was subtracted. These figures indicate that in the presence of nicotinic acid there was actually a greater conversion of tryptophan to N^1-methylnicotinamide than when nicotinic acid was lacking. The increase in N^1-methylnicotinamide due to the addition of nicotinic acid was greatest in the presence of tryptophan and least in the absence of tryptophan. At the 20 mg. level of tryptophan this increase was also greater in the presence of pyridoxine than in its absence, but this was not so at the 100 mg. level of L-tryptophan.

TABLE IV—*Isolation of*

			Effect of tryptophan				
			Nicotinic acid		Pyridoxine		Nicotini
			+	−	+	−	+
2nd wk.	Tryptophan	Difference, %	381.2	628.6	545.9	450.8	31.9
		P*	0.004	<0.0005	<0.0005	0.0028	0.25
	Nicotinic acid	Difference, %	79.5	86.0	78.1	86.4	17.2
		P	<0.0005	0.0023	0.0033	<0.0005	0.097
	N^1-Methylnicotinamide	Difference, %	204.2	39.4	159.4	126.6	74.6
		P	0.0023	0.018	0.0046	0.01	0.031
	Xanthurenic acid	Difference, %	220	0	0	220	−220
		P	0.041			0.041	0.041
3rd wk.	Tryptophan	Difference, %					3.1
		P					>0.45
	Nicotinic acid	Difference, %					8.3
		P					0.300
	N^1-Methylnicotinamide	Difference, %					116.7
		P					<0.0005
	Xanthurenic acid	Difference, %					−420
		P					<0.0005
After 3rd wk.†	Tryptophan	Difference, %					535.2
		P					0.02
	Nicotinic acid	Difference, %					47.6
		P					0.083
	N^1-Methylnicotinamide	Difference, %					18.5
		P					0.300
	Xanthurenic acid	Difference, %					−6083.5
		P					<0.0005

* P = probability of chance outcome.

† Urine was collected for 24 hours following a dose of 100 mg. of L-tryptophan.

The presence of nicotinic acid also resulted in a greater conversion of tryptophan to xanthurenic acid in pyridoxine deficiency, and during the 2nd week there was no detectable excretion of xanthurenic acid in the group which did not receive nicotinic acid. During the 3rd week both groups converted more tryptophan to xanthurenic acid, but there was almost twice as much xanthurenic acid excreted, as well as a greater amount of tryptophan, by the group receiving nicotinic acid.

Effect of Succinylsulfathiazole—Succinylsulfathiazole had no significant effect on the excretion of tryptophan. It had a variable effect, which is not statistically significant, on the percentage conversion of tryptophan to nicotinic acid, but the recovery of added nicotinic acid was 29.6 and 35.5 per cent less, and during the 3rd week there was a striking failure to recover any nicotinic acid as such. When 100 mg. of L-tryptophan were fed, succinylsulfathiazole surprisingly gave a greater conversion of tryptophan to nicotinic acid and also a greater recovery of added nicotinic acid, although the latter is not statistically significant ($P = 0.069$). Succinylsulfathiazole had no depressing effect, either on the conversion of tryptophan to N¹-methylnicotinamide or the recovery of added nicotinic acid in the form of N¹-methylnicotinamide; in fact, when 100 mg. of L-tryptophan

ffects on Urinary Excretion

ect of pyridoxine			Effect of nicotinic acid						Effect of sulfasuxidine	
cid	Tryptophan		Pyridoxine		Tryptophan		Sulfasuxidine		Nicotinic acid	
−	+	−	+	−	+	−	+	−	+	−
37.9	38.5	18.1	−28.2	−24.9	−31.1	4.3	−25.4	−37.9	−7.9	−23.3
0.083	0.094	0.365	0.106	0.225	0.069	>0.45	0.216	0.061	0.405	0.188
1.4	9.6	14.1	70.3	47.4	57.0	62.7	17.4	59.7	−29.6	−4.2
>0.45	0.097	0.294	0.0042	<0.0005	<0.0005	0.04	0.061	0.0013	0.0045	0.312
22.2	66.1	45.1	257.1	150.0	299.3	83.0	362.6	379.4	−13.1	−10.0
0.086	0.012	0.249	0.0016	0.006	<0.0005	0.147	<0.0005	<0.0005	0.230	0.154
0	−220	0	0	220	220	0	0	0	0	0
	0.041			0.041	0.041					
57.3			−13.5	31.9			−8.1	−13.5	−23.6	−28.1
0.139			0.326	0.258			0.299	0.326	0.125	0.156
1.4			66.4	55.8			−2.4	66.4	−35.5	10.1
0.375			0.0097	<0.0005			0.427	0.0097	0.028	0.059
−13.1			415.4	106.7			286.3	415.4	−11.8	17.6
0.277			<0.0005	0.019			<0.0005	<0.0005	0,219	0.061
−216.6			0	93.9			0	0	0	0
<0.0005				0.214						
−38.5			114.7	−79.2			7.0	114.7	−20.8	59.0
0.202			0.067	0.073			>0.45	0.067	0.339	0.202
12.6			75.9	34.2			96.7	75.9	34.8	20.5
0.144			0.007	0.146			0.0033	0.007	0.069	0.047
64.5			305.6	462.8			189.3	305.6	5.3	47.5
0.087			<0.0005	0.02			0.0023	0.0007	0.400	0.103
−833.3			0	114.7			0	0	0	0
<0.0005				0.047						

were fed, the excretion values were higher, but the differences were not statistically significant.

Effect of Time—The only fundamental differences in the urinary excretion between the 2nd and 3rd weeks, *i.e.* the effect of time, are an increase in the formation of xanthurenic acid and an increase in the conversion of tryptophan to nicotinic acid in the presence of succinylsulfathiazole, which is on the border line of statistical significance ($P = 0.059$).

Comparison of Tryptophan Levels—It is worth while to compare the results obtained with an adequate amount of tryptophan (20 mg.) with those of an excess of tryptophan (100 mg.). At the higher level of tryptophan there was a greater percentage of tryptophan excreted except in the pyridoxine-deficient group receiving nicotinic acid, in which there was a very large per cent of tryptophan excreted as xanthurenic acid, and, as at the lower level, this xanthurenic acid excretion was greater than the combined excretion of tryptophan and xanthurenic acid when nicotinic acid was not added. In both pyridoxine-deficient groups there was a greater per cent of tryptophan converted to xanthurenic acid at the 100 mg. level than at the 20 mg. level.

As the amount of tryptophan was increased from 1.8 to 20 mg. and 100 mg., there was an increase in the actual amount of nicotinic acid excreted, but the percentage conversion was progressively less. The same was true for N^1-methylnicotinamide with the exception that when pyridoxine was present the per cent conversion of tryptophan to N^1-methylnicotinamide was the same at both levels of added tryptophan. The recovery of added nicotinic acid in the form of N^1-methylnicotinamide ranged from only 16.7 to 56.5 per cent at the 20 mg. level of tryptophan, while at the 100 mg. level of tryptophan the recovery was in considerable excess of the amount added, with the range 181.0 to 198.0 per cent.

DISCUSSION

The apparent interchangeability of nicotinic acid and tryptophan in promoting growth has been demonstrated (1–7) only with diets containing marginal amounts of tryptophan. However, the present study shows that, while tryptophan can entirely replace nicotinic acid, nicotinic acid cannot replace tryptophan, which is one of the ten animo acids essential for the growth and maintenance of rats. Thus, in the absence of added tryptophan (only 1.8 mg. per day) nicotinic acid had no effect on the survival time.

The failure to obtain a beneficial effect of pyridoxine on gain in weight is surprising, especially since forced feeding should have exaggerated the deficiency of pyridoxine. The answer does not seem to lie in the duration of the experiment, for definite gross symptoms of pyridoxine deficiency were obtained in all animals not receiving this vitamin, and the excretion of xanthurenic acid confirmed the existence of the metabolic defect associated with pyridoxine deficiency. The seeming ability of the tryptophan-deficient animals to show a gain in weight for the 1st week which was not significantly less than that of the tryptophan-supplemented animals may have been due to complication with bloat.

In a previous publication from this laboratory (3) it was postulated that the interrelationship between tryptophan and nicotinic acid when the dietary intake of both is low may be analogous to the metabolic interrelation of methionine and choline. Singal and coworkers (9) suggested a similar analogy, the methionine-cystine relationship, to describe the interchangeable rôle of tryptophan and nicotinic acid. The large increase in the urinary excretion of nicotinic acid and its derivatives following the addition of tryptophan to various diets in the albino rat (4, 5, 8, 9), cotton-rat and horse (24), pig (25), man (26, 27), and dog (28) has been interpreted by these investigators to indicate that tryptophan is the dietary precursor of niacin. Although the evidence is circumstantial, the present study supports this conclusion as the most probable explanation of the increased urinary excretion of niacin following tryptophan administration.

The following facts, that (1) tryptophan supplementation resulted in increased urinary excretion of nicotinic acid and N^1-methylnicotinamide on a diet extremely low in nicotinic acid, (2) this excretion continued for over 3 weeks, (3) there was an increase in nicotinic acid and N^1-methylnicotinamide excretion when tryptophan was increased, (4) over 200 per cent of added nicotinic acid was recovered when 100 mg. of L-tryptophan were given, indicate that tryptophan is being converted into nicotinic acid and N^1-methylnicotinamide rather than that tryptophan is sparing or mobilizing the body's store of this vitamin. To provide direct proof for the probable conversion of tryptophan to niacin a tracer experiment, with tryptophan labeled with isotopic carbon or nitrogen, has been suggested (29, 30).

Ellinger and Benesch (31) have reported that succinylsulfathiazole diminished the urinary output of N^1-methylnicotinamide by an average of 60 per cent in man and interpreted this as demonstrating a biosynthesis of nicotinamide in the gut. Later (32) Ellinger reported that this drug also reduced the excretion of N^1-methylnicotinamide in the rat. Najjar *et al.* (33) found no reduction in the excretion of N^1-methylnicotinamide upon the administration of succinylsulfathiazole, and suggested that the difference may have been due to the nature of the basal diets used. In this study the results with succinylsulfathiazole indicate that the conversion of tryptophan to nicotinic acid and N^1-methylnicotinamide is probably not dependent upon the synthetic activities of intestinal microrganisms.

The only significant effect of pyridoxine on niacin excretion observed in this study was an increase in N^1-methylnicotinamide when nicotinic acid and 20 mg. of tryptophan were also added, but not when nicotinic acid was absent, and at the 100 mg. level of tryptophan there was no effect of pyridoxine even when nicotinic acid was present. These observations suggest that the synthesis of N^1-methylnicotinamide from tryptophan was increased by the addition of nicotinic acid, and this increase was greatest in the presence of pyridoxine at the 20 mg. level of tryptophan, but any beneficial effect of pyridoxine is probably dependent upon the presence of an adequate amount of nicotinic acid.

Rosen, Huff, and Perlzweig (14) reported that pyridoxine deficiency affects adversely the transformation of tryptophan to nicotinic acid in the rat. As the pyridoxine deficiency became more acute, as measured by the increase in xanthurenic acid, they found a progressive decrease in the excretion of N^1-methylnicotinamide. In the present study there was a greater excretion of xanthurenic acid during the 3rd week than during the 2nd week, but this was not at the expense of a decrease in N^1-methylnicotinamide. Moreover, these investigators pointed out that the formation of xanthurenic acid is not directly related to the decreased formation of niacin. Furthermore, they found that the administration of kynurenine, kynurenic acid, and xanthurenic acid to rats on a complete diet failed to produce increased amounts of N^1-methylnicotinamide in the urine, and concluded, therefore, that the metabolic course from tryptophan to nicotinic acid in the rat is not via the kynurenine pathway. These authors also found that the mechanism for methylating nicotinamide is not impaired in a vitamin B_6-deficient rat, nor is there an abnormal rate of destruction of N^1-methylnicotinamide.

Schweigert and Pearson (15) have also reported that when tryptophan is fed pyridoxine-deficient rats and mice have a greatly reduced ability to convert this amino acid to nicotinic acid and N^1-methylnicotinamide. Their data reveal that when no tryptophan was added to the basal ration (which contributed 3.1 mg. of tryptophan per gm.) the excretion of N^1-methylnicotinamide and nicotinic acid in micrograms per rat per day was greater for the animals receiving pyridoxine (93 and 23.4, respectively) than for the animals not receiving it (48 and 9.9), while on the basis of micrograms per gm. of food the excretion was not significantly different, 9.3 and 2.4, respectively, for those receiving pyridoxine, and 9.9 and 2.1 for those not receiving it. Thus, on the basal diet pyridoxine had no effect on the conversion of tryptophan to nicotinic acid, 0.14 per cent for the pyridoxine-supplemented group and 0.11 per cent for the pyridoxine-deficient group, while for N^1-methylnicotinamide the conversion for the pyridoxine-supplemented group was only 0.54 per cent, whereas it was 0.64 per cent for the pyridoxine-deficient group. Only when 100 mg. of DL-tryptophan were fed in addition to that in the diet was there a definite increase in N^1-methylnicotinamide and nicotinic acid in favor of the pyridoxine-supplemented group.

The explanation for the difference in the results of these investigators (14, 15) and of the present study may lie in the difference in experimental techniques. Their basal rations which contained an adequate amount of tryptophan, 4.5 mg. per gm. (14) and 3.1 mg. per gm. (15), were fed *ad libitum*. In the study by the Duke group (14) a complete cessation of growth was reported for the pyridoxine-deficient rats, while in that of the Texas group (15) the deficient animals gained only 10 gm. per week, whereas the pyridoxine-supplemented rats grew at a rate of 20 gm. per week. Their data indicate that the food consumption of the deficient group was also about half of that for the pyridoxine-supplemented rats.

A definition of nutritional adaptation has been given by Mitchell (34), who has reworded the theorem of Le Chatelier to apply to animal life in its relation to the food supply: "If an animal in equilibrium with its food supply (meaning a well-nourished animal) is subjected to nutritional stress, such as an inadequate (or an excessive) supply of one or more of the essential nutrients, the animal will react in such a way as to minimize, as far as possible, or to undo entirely the effects of the nutritional stress." One of the first and most common adjustments an animal makes to an inadequate diet, regardless of the type of deficiency, is a reduction of food consumption. Experiments intended to determine the specific effect and rôle of any particular nutrient must be designed to avoid complication by a general response of lowered food consumption and the common effect associated with generalized undernutrition. Equalizing the food intakes of comparative animals by paired feeding differentiates between the specific effect of any particular dietary treatment and the general effect associated with poor appetite. Forced feeding, when feasible, is even more desirable because it not only completely eliminates complication by generalized undernutrition, but also accentuates the inherent effect of any dietary modification by blocking the most common mechanism of adaptation to nutritional stress. Another advantage of forced feeding is a decrease in the time required to produce an acute deficiency, illustrated in this study by the early symptoms and death of the tryptophan-deficient animals.

In studies of the urinary excretion of nicotinic acid and its derivatives it is well to consider that our knowledge of the end-products of nicotinic acid metabolism is incomplete. Perlzweig and Huff (35) have stated that "the excretion of F_2 [N^1-methylnicotinamide] represents primarily the resultant of the rates of two or more metabolic reactions involving niacin: its methylation to F_2 and the subsequent conversion of F_2 to products as yet unknown." A new metabolite of nicotinamide, identified as the product of oxidation *in vitro* of N^1-methylnicotinamide by the quinine-oxidizing enzyme of rabbit liver, has in fact been isolated from human urine after nicotinamide administration, and identified as the 6-pyridone of N^1-methylnicotinamide (36, 37). Knox and Grossman believe that this pyridone is excreted in an amount at least comparable to that of N^1-methylnicotinamide. The appearance of a considerable amount of an acid-labile nicotinic acid precursor in the urine of rats receiving large amounts of tryptophan suggests that it may be an intermediate in the synthesis of nicotinic acid (9). Incomplete data obtained in this study indicate that this substance is also produced when only 20 mg. of tryptophan are given. The physiologic significance of these compounds, which were not measured in this study, is at present not known.

The present study shows that, while significant differences were observed in urinary excretion values, the only significant difference in gain in weight was that obtained during the 1st week by feeding 2 per cent succinylsulfathiazole. Thus, under the present experimental conditions, there was no relationship between the gain in body weight and the urinary excretion of the compounds tested.

SUMMARY

An acid-hydrolyzed casein diet deficient in tryptophan, pyridoxine, and nicotinic acid was fed directly into the stomach. 60 young rats were divided into ten groups of six each. Eight groups provided all possible combinations of the following supplements: 20 mg. of L-tryptophan, 0.2 mg. of pyridoxine, and 0.2 mg. of nicotinic acid. Two groups received 2 per cent succinylsulfathiazole. Individual urinary collections were made for 24 hours during the 2nd and 3rd weeks and after an oral dose of 100 mg. of L-tryptophan during the 4th week. The urine was analyzed for free tryptophan, nicotinic acid, N^1-methylnicotinamide, and xanthurenic acid.

Statistical examination of the data indicated no significant effect of any of the supplements upon gain in weight, even though definite symptoms of pyridoxine and tryptophan deficiency were observed. The presence of 2 per cent succinylsulfathiazole in the diet resulted in a greater gain in body weight during the 1st week, but not in subsequent weeks.

The tryptophan-deficient animals progressively exhibited bloat, diarrhea, and convulsions with screeching. Death occurred as early as the 6th day, and all animals were dead after 13 days of a tryptophan deficiency. Histologically, there was indication of fatty degeneration of the cells in the liver.

The addition of 20 mg. of L-tryptophan increased the excretion of nicotinic acid from 78 to 86 per cent, regardless of the presence or absence of pyridoxine or nicotinic acid. The increase in N^1-methylnicotinamide due to the addition of tryptophan was considerably greater when nicotinic acid was present (204 per cent) than when it was lacking (39 per cent). The recovery of added nicotinic acid in the form of N^1-methylnicotinamide was 181 to 198 per cent when 100 mg. of L-tryptophan were fed. This indicates that when nicotinic acid was present there was a greater conversion of tryptophan to N^1-methylnicotinamide than when nicotinic acid was lacking.

Pyridoxine has no significant effect on the conversion of tryptophan to nicotinic acid and N^1-methylnicotinamide. The presence of nicotinic acid resulted in a greater formation of xanthurenic acid in pyridoxine deficiency.

The results obtained with 2 per cent succinylsulfathiazole in the diet indicate that the conversion of tryptophan to nicotinic acid and N^1-methylnicotinamide is probably not dependent upon the synthetic activities of intestinal microorganisms.

These observations contribute additional evidence in support of the conclusion that tryptophan is a dietary precursor of niacin as the most logical explanation for the greater urinary excretion of nicotinic acid and N^1-methylnicotinamide by tryptophan-supplemented animals.

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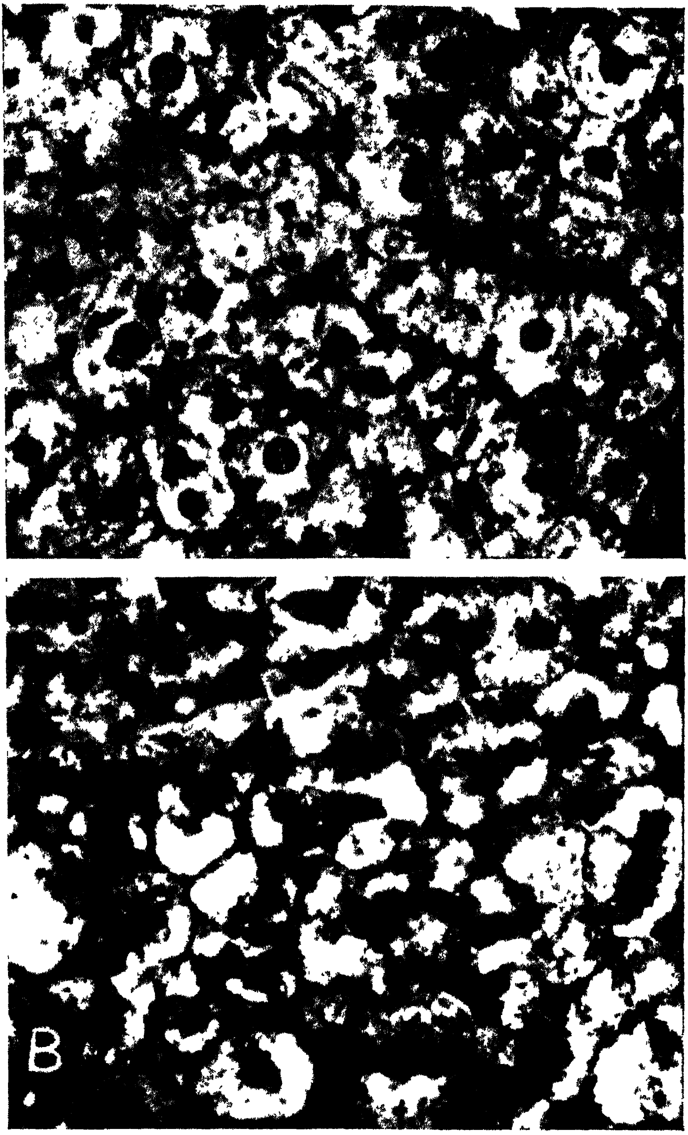
EXPLANATION OF PLATE 1

FIG. 1. Histological sections of the liver (× 500); hematoxylin-eosin stain of Harris. *A*, normal; *B*, tryptophan-deficient.



(Spector: Interrelation of vitamin supplements)

A MICRO PHOTOFLUOROMETER

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A need arose for a fluorometer sufficiently sensitive to measure the amounts of riboflavin and thiamine in a few hundredths of a ml. of blood or blood serum. There are approximately 1 and 0.25 millimicrograms of riboflavin and thiamine respectively in 20 c.mm. of blood serum, an amount conveniently obtainable from the finger. Conventional commercial fluorometers require 100- to 1000-fold greater quantities of these vitamins for adequate measurement. Friedemann and Frazier (1) have reported the use of a more sensitive fluorometer, but the instrument has not been described. Hinton (2) used a very sensitive galvanometer in conjunction with the Spekker fluorometer and was able to measure as little as 1 millimicrogram of thiamine with a reproducibility of 20 per cent. Special capillary cells were employed.

It has been found easily possible to increase the sensitivity (and stability) of a commercial fluorometer 1000-fold by substituting a "multiplier" phototube for the phototube originally present. With this modified instrument, 0.1 millimicrogram of riboflavin (10^{-10} gm.) in 0.5 ml. of solution can be measured with a precision of 5 per cent. An instrument embodying the same principles is now available commercially.[1]

Description will be given of the changes made to increase the sensitivity and stability of the instrument and of the alterations necessary to decrease the optical blank, the last being more difficult to accomplish.

Increasing the Sensitivity—The phototube of the Coleman photofluorometer, model 12, was replaced by an RCA multiplier phototube, No. 1P21.[2] A standard eleven prong tube socket was substituted for the original socket, and was carefully oriented to present the photo surface directly to the light beam. (The sensitive area on the multiplier tube is only a few mm. wide.) The eleven well insulated leads from the socket were carried out of the tube housing and instrument box. The tube housing was carefully sealed with black tape to exclude indirect light. Except for the mercury vapor lamp and fan, the original electrical equipment in the box was disconnected and not used.

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[1] Farrand Optical Company, Inc., Bronx Boulevard and East 238th Street, New York 66, New York.

[2] There is a series of RCA multiplier phototubes. No. 1P21 is a selected tube of high sensitivity and stability, and is, therefore, rather expensive. No. 931-A, which is a similar but unselected tube, selling for considerably less, is not recommended for the present purpose. As far as the author is aware, none of the other phototubes of the series, *i.e.* those with other photo surfaces or envelopes, is available in as highly a selected form as No. 1P21.

The electrical circuit employed is very simple (Fig. 1). The output of the tube is led without further amplification to the galvanometer (model 34034 of the Rubicon Company, Philadelphia; sensitivity about 1 microampere, full scale). A coarse and fine sensitivity adjustment is provided which permits a 100-fold range in sensitivity. The fixed resistance between the two inexpensive radio potentiometers (Fig. 1) is to prevent the sensitivity from being reduced to less than 1 per cent of the maximum. At this lowest sensitivity the galvanometer will go off scale when the phototube output is greater than about 0.1 milliampere, which is a safe upper limit to prevent damage to the tube.

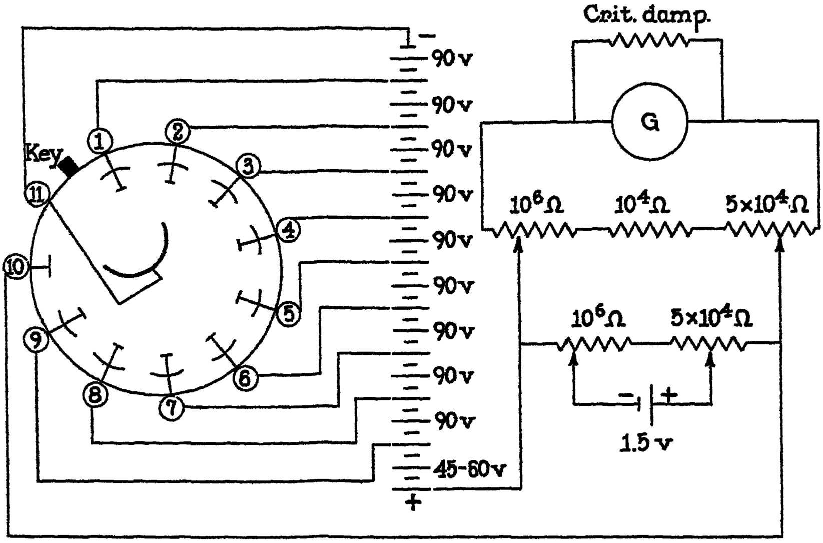

FIG. 1. Wiring diagram for multiplier phototube

The choice of batteries rather than a transformer for voltage supply is strongly recommended for the sake of simplicity, stability, and freedom from trouble. The voltage must above all be constant. The initial cost of batteries is low and, since the drain is negligible, replacement is only necessary after a year or two of service. Burgess 45 volt B batteries, No. W30BPX, and especially Eveready 30 volt Minimax batteries, No. 413, are conveniently small in size, and both have been used successfully. As a precaution, the individual electrodes have been disconnected when not in service, with either gang switches or multiwire cable connectors. The necessity for prevention of electrical leakage with the high voltages used should be taken into account in the construction. However, the inherent design of the phototube and the circuit used is such that electrical leaks are of relatively minor significance in contrast with the troublesomeness of the usual phototube-high impedance circuit.

The battery used for the dark current adjustment may be kept in the circuit continuously, since there is a negligible drain through the high resistance of the shunt.

Because of the great sensitivity of the phototube, it is necessary during readings to cover the tube containing the sample to prevent outside light from entering. Indeed, the external room light must be kept subdued to prevent large galvanometer deflections due to reflections when the tubes are inserted. Red light, to which the phototube is relatively insensitive, may be used conveniently in the room.

Reduction of Optical Blank—As the sensitivity of the instrument is increased, the galvanometer deflection with a blank tube of distilled water increases and limits the useful sensitivity obtainable. This blank is chiefly due to fluorescence of the various components of the optical system; *viz.*, lenses, light filters and the sample tube itself. Soft glass gives off a weak fluorescence, especially with shorter exciting wave-lengths. This can be largely eliminated by substituting Pyrex tubes. The two original lenses in the instrument also have a slight fluorescence which can be eliminated by replacing them with equivalent Pyrex or quartz lenses. This improvement is, however, insufficient to warrant the trouble under ordinary circumstances. It is more difficult to eliminate fluorescence of the light filters. The effect of their fluorescence can, however, be reduced by reducing the scattered light within the system. Two changes proved helpful in this regard. In the path of the exciting light beam was fixed a wooden baffle which was so carved that only the central portion of the tube was illuminated (Fig. 2). The wood was blackened with India ink. To reduce scattered light further, the inner chamber which surrounds the sample tube was lined with black cloth, which is much less reflecting than the original smooth, blackened metal surface. After these changes were made, the optical blank was measured with the light filter combination appropriate for riboflavin. (The primary filter, Coleman B_2, consists of Corning glass Filters 5113 and 3389, the latter facing the light source. The secondary filter, Coleman PC2, is Corning glass Filter 3486.) A 19 mm. diameter soft glass tube filled with redistilled water gave a galvanometer deflection equivalent to that obtained with 3 millimicrograms of riboflavin in 8 ml.

By substituting a Pyrex tube, the blank was reduced to just half of this value.

The ratio of riboflavin reading to optical blank was still further improved by reducing the sample volume and using smaller Pyrex tubes (9 mm. outside diameter × 10 cm.) in an adapter (Fig. 2). With this arrangement, which required no more than 0.5 ml. of solution, the optical blank was equivalent to only 0.2 millimicrogram of riboflavin per 0.5 ml. The reduction in volume has the further general advantage of increasing the concentration of measured substances relative to the blank fluorescence of the reagents used.

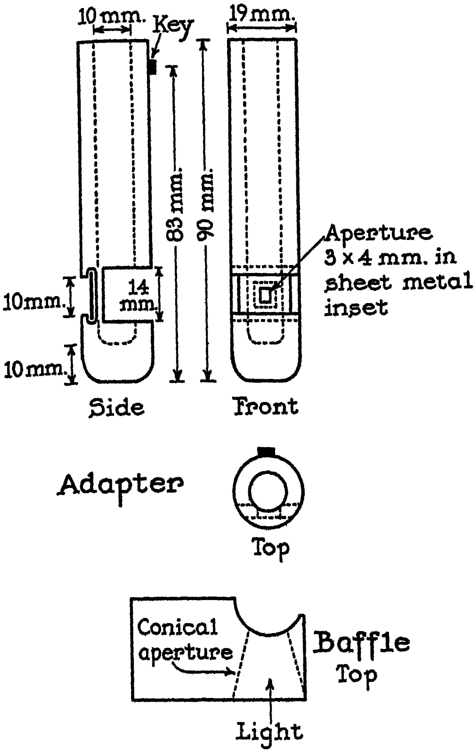
10 mm.
Key
19 mm.
83 mm.
90 mm.
Aperture
3 x 4 mm. in
sheet metal
inset
10mm.
14
mm.
10mm.
Side
Front
Adapter
Top
Conical
aperture
Baffle
Top
Light

FIG. 2. Adapter and baffle for micro photofluorometer

Greater difficulty was encountered in reducing the optical blank with the filter combination used for the measurement of thiochrome, and other substances requiring the 365 mμ mercury band for excitation (the condensation products of diphosphopyridine nucleotide or N^1-methylnicotinamide with ketones (3), pyridoxine derivatives (4), xanthopterin and related products, quinine, etc.). The usual filter combination consists of Corning glass Filter 587 as the primary (Coleman, B1) and Corning Filters 3389 and 4308 as the secondary (Coleman, PC1). The Corning Filter 3389 is itself quite fluorescent. This can be decreased by inserting a film of Wratten gelatin filter, No. 2A (Eastman Kodak Company), between Filters 3389 and 4308 with Filter 3389 faced toward the phototube. Filter 2A, gelatin film, prevents the ultraviolet light from reaching Filter 3389.

TABLE I

Riboflavin Measurements with Micro Photofluorometer*

8 ml. volume		0.5 ml. volume	
Riboflavin present	Riboflavin found	Riboflavin present	Riboflavin found
millimicrograms	*millimicrograms*	*millimicrograms*	*millimicrograms*
0.53	0.54	0.106	0.107
0.53	0.59	0.106	0.105
0.53	0.55	0.106	0.108
		0.106	0.106
Average 0.53	0.56	0.106	0.106
1.99	2.00	0.53	0.54
1.99	2.00	0.53	0.52
1.99	2.07	0.53	0.51
		0.53	0.51
Average 1.99	2.02	0.53	0.52
20.8	20.7	1.99	1.98
20.8	20.9	1.99	2.04
20.8	21.0	1.99	2.14
		1.99	2.05
Average 20.8	20.9	1.99	2.05

* The riboflavin was measured in the usual manner by comparing the initial fluorescence with the increase in fluorescence when an internal riboflavin standard was added.

Unfortunately, Filter 2A is itself slightly fluorescent, apparently owing to the gelatin; hence, the combination is still imperfect although much improved. So modified, the optical blank with 0.5 ml. of redistilled water in a 9 mm. Pyrex tube was equivalent to the fluorescence of the thiochrome from 0.6 millimicrogram of thiamine in 0.5 ml. Although this is not as low a blank as is desirable, the fluorescence of reagents and other substances likely to be present is larger than the optical blank, which becomes, therefore, of secondary importance.

Performance of Micro Fluorometer—Table I illustrates the measurement of 0.1, 0.5, and 2 millimicrogram quantities of riboflavin in a volume of 0.5 ml., and 0.5, 2.0, and 20 millimicrogram quantities in 8 ml. The precision of measurement is seen to be quite adequate, and compares favorably with that of macro instruments which require 1000-fold larger samples. At full sensitivity, with the adapter, 0.1 millimicrogram of riboflavin in 0.5 ml. (1 part in 5 billion) gave a galvanometer deflection of 6 divisions. At one-third sensitivity, without the adapter, 0.5 millimicrogram of riboflavin in 8 ml. (1 part in 16 billion) gave a deflection of 8 divisions. Thus, if the optical blank could be further reduced, it would be possible to measure riboflavin at a dilution of 1 to 50 billion. The response with thiochrome is nearly 20 times greater than with riboflavin; hence, it could conceivably be measured at a dilution of 1 to a trillion if the optical blank were sufficiently reduced.

DISCUSSION

Fluorimetry is inherently a much more sensitive analytical tool than colorimetry for measuring *concentrations* of substances. In colorimetric procedures a significant percentage of the light must be absorbed, and this is determined almost entirely by the concentration of the substance and the length of light path. In measurement of a fluorescent substance the sensitivity is proportional not only to the concentration of the substance and the length of light path, but also to the intensity of illumination and the sensitivity of the photometer. There are limitations to the increase in illumination permissible with light-sensitive materials, but the sensitivity of the photometer can be greatly increased. There are few substances which can be measured colorimetrically at concentrations less than 1 part per 10 million with a reasonable light path. This is a concentration 2000 times stronger that that of the riboflavin solution measured above, and this vitamin is not an exceptionally fluorescent substance.

SUMMARY

A micro fluorometer is described which is 100 to 1000 times more sensitive than existing commercial instruments, and which is capable of measuring as little as 0.1 millimicrogram of riboflavin, for example, with a precision of 5 per cent, and larger quantities with at least the precision attainable with macro fluorometers.

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A SIMPLE QUANTITATIVE CHEMICAL METHOD FOR ESTIMATING γ-GLOBULIN IN HUMAN SERUM*

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Both sodium sulfate and potassium phosphate have been employed for the quantitative estimation of globulin fractions in human serum (1–6). With these procedures, the quantities of the various globulins are determined by subtractive methods after precipitation of portions of total globulin. Also the volumes of salt solution added per ml. of serum are large. The method outlined here for estimation of γ-globulin departs from these procedures in that it entails the direct estimation of the protein content of the precipitate obtained by adding a small volume of saturated ammonium sulfate to undiluted serum. Certain characteristics of the fraction precipitated have been examined. In both normal and pathological sera, the quantity of protein present in this fraction was found to correlate well with values estimated electrophoretically for γ-globulin in the same samples.

Method

Reagents—

1. Saturated ammonium sulfate brought to pH 7.0 with ammonium hydroxide.
2. 0.33 saturated ammonium sulfate (pH 7.0).
3. The dilute biuret reagent of Weichselbaum (7).

Technique—To 1.0 ml. of fresh serum in a 12 ml. centrifuge tube is added 0.5 ml. of saturated ammonium sulfate drop by drop, the precipitate being shaken *thoroughly* after addition of each 0.1 ml. The precipitate generally will disappear after shaking until more than 0.3 ml. of salt solution is added. The suspension is placed at 4° overnight. It is then centrifuged for 30 minutes at 3000 R.P.M. in an angle centrifuge. The pale yellow, clear supernatant fluid is removed by aspiration and discarded. The packed precipitate is *finely emulsified* in the tube with a stirring rod after addition of 3.0 ml. of 33.3 per cent saturated ammonium sulfate. The tube is recentrifuged for 30 minutes at 3000 R.P.M. and the clear, colorless supernatant removed. The precipitate is dissolved by stirring and shaking in 10 ml. of

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0.85 per cent sodium chloride. To 5.0 ml. of this slightly opalescent colorless fluid are added 5.0 ml. of the biuret reagent. The protein content is measured in a Coleman model 11 universal spectrophotometer, with 7 mm. cuvettes and at a wave-length setting of 555 mμ.

Except for the initial refrigeration of the precipitate, all steps are performed at room temperature. If the serum is centrifuged immediately after the initial addition of ammonium sulfate, the protein content of the precipitate frequently is 10 to 20 per cent lower than that of a sample permitted to stand overnight in the refrigerator. When the serum has a high lipide content, the precipitate redissolved in saline may appear turbid even after the biuret reagent has been added. In such instances the lipide is extracted with ether immediately before the spectrophotometric reading.

Repeated protein determinations on a standard solution of rabbit serum with the biuret technique and with a micro-Kjeldahl method (8) gave agreement within ±2 per cent. The small amount of ammonium sulfate present in the precipitate redissolved in saline was not found to affect significantly the development of the biuret color reaction. In a few experiments in which sera were kept frozen for as long as 4 weeks, the quantities of precipitate were equal to those obtained with fresh sera.

Experimental Observations

Accuracy of Method—The quantity of washed precipitate (Fraction G.G.-33.3) obtained with this technique was determined in triplicate on the sera of ten normal subjects (Table I). The standard deviation of the method was 0.021 gm., which gave a coefficient of variation of ±2.7 per cent.

Protein-Bound Phosphorus Content—Aliquots of protein obtained by dialysis of whole serum, of the supernatant fraction after precipitation with 33.3 per cent saturated ammonium sulfate, and of the washed 33.3 per cent precipitate were digested with perchloric acid (9) and the phosphorus content determined spectrophotometrically (10). With the mean values obtained from determinations on seven separate sera and their fractions, the protein-bound phosphorus content, as mg. of P per gm. of protein, is as follows: whole serum 2.02, supernatant 1.75, and washed precipitate 0.24. The protein-bound phosphorus content of the precipitate, while low, is somewhat greater than that of electrophoretically isolated γ-globulin as determined by Blix and coworkers (11).

Antibody Content—Enders (12) has shown that many of the antibodies present in human sera are concentrated in Cohn's Fraction II, which consists almost entirely of γ-globulin (13). A few antibodies, however, such as those to the typhoid O antigen and isoagglutinins to blood types A and B were found to be concentrated in Fraction III_1, which consists mostly of β- and γ-globulins. Using methods of antibody measurement similar to those of Enders, we determined the concentration in whole serum and in the two fractions obtained with ammonium sulfate. Each fraction after dialysis was brought to the protein content it possessed in the original serum. The results (Table II) indicate that diphtheria antitoxin[1] and

TABLE I

Triplicate Determinations of 33.3 Per Cent Saturated Ammonium Sulfate Precipitate (Washed) in Ten Normal Human Sera

Expressed in gm. of precipitate per 100 ml. of serum.

Serum	Determination 1	Determination 2	Determination 3	Mean
R. B.	0.661	0.666	0.668	0.665
B. G.	0.846	0.828	0.850	0.841
R. G.	1.082	1.210	1.104	1.132
V. D.	0.752	0.742	0.718	0.737
H. A.	0.980	0.994	1.000	0.991
M. N.	1.070	1.103	1.070	1.081
V. J.	0.738	0.722	0.738	0.733
B. L.	1.037	1.055	1.055	1.049
J. T.	0.679	0.661	0.668	0.669
A. D.	1.077	1.045	1.037	1.053

Standard deviation of the method, 0.021 gm.; coefficient of variation, ±2.66 per cent.

TABLE II

Antibody Content in Whole Serum and in Serum Fraction

The antibody content of each fraction was determined by using a protein content per ml. identical with that which this fraction possessed in the original serum. Antibody titers are expressed as reciprocals of the last dilution of serum or fraction giving 2+ agglutination with the exception of diphtheria antitoxin which is measured by the rabbit skin test, the values being in units per ml.

Antibody	Source	Antibody concentration per ml.		
		Whole serum	Fraction G.G.33.3	Supernatant
Diphtheria antitoxin	Pooled normal sera		0.075	0.018
" "	Convalescent patient	0.32	0.17	0.14
Typhoid H	Immunized subject	5120	5120	2560
" O	" "	160	0 (<20)	80
Isoantibody against group A cells	Normal subject	64	0 (<8)	64
Heterophil antibody	Patient with infectious mononucleosis	320	80	320

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[1] Diphtheria toxin and antitoxin were furnished by the Lederle Laboratories Division, American Cyanamid Company.

typhoid H antibody are concentrated in Fraction G.G.33.3. However, isoagglutinins to group A cells, the heterophil antibody, and typhoid O antibody were not concentrated in this fraction.

Electrophoretic Composition of Fractions—Analyses were made in a Tiselius apparatus equipped with a Longsworth schlieren scanning device.

TABLE III

Electrophoretic Analysis of Whole Serum and of Fractions Obtained with 33.3 Per Cent Saturated Ammonium Sulfate

The values are expressed in gm. of protein per 100 ml. of serum and as per cent of the total serum protein.

Sample	Total protein	Albumin		Globulin					
				α_1 and α_2		β_1 and β_2		γ_1 and γ_2	
Pooled normal serum									
	gm.	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
Whole serum	7.79	4.60	59	1.17	15	0.93	12	1.09	14
Supernatant*	5.91	4.20	71	1.00	17	0.59	10	0.12	2
Fraction G.G.33.3	1.04	0	0	0.17	16	0.11	11	0.76	73
Wash of Fraction* G.G.33.3	0.84	0.21	25	0.19	22	0.06	7	0.39	46
Whole serum	7.67	4.75	62	1.07	14	0.84	11	0.96	13
Supernatant*	6.42	4.43	69	1.09	17	0.90	14	0	0
Fraction G.G.33.3	0.97	0.01	1	0.05	5	0.13	14	0.78	80
Wash of Fraction* G.G.33.3	0.28	0.09	31	0.03	12	0.03	12	0.13	45
Serum from case of arthritis									
Whole serum	7.92	3.64	46	1.19	15	0.79	10	2.30	29
Supernatant*	5.20	3.27	63	1.35	26	0.57	11	0	0
Fraction G.G.33.3	2.07	0	0	0.06	3	0.29	14	1.72	83
Wash of Fraction* G.G.33.3	0.65	0.23	35	0	0	0.08	12	0.34	53

* Corrected for the volume increase resulting from the addition of ammonium sulfate solution.

Runs were made at 1.5°, with a veronal buffer at pH 8.4 to 8.6, an ionic strength of 0.1, and a cell protein concentration of 1.5 gm. per cent. In the various studies here and in the subsequent section, the mobilities of the various fractions exhibited the following ranges ($\times 10^{-5}$ sq. cm. per volt per second): γ-globulin, 0.8 to 1.3; β-globulin, 2.8 to 3.7; α-globulin, 4.1 to 5.5; albumin, 6.0 to 6.7.[2]

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[2] Electrophoretic studies were carried out by Dr. Emil L. Smith with the technical assistance of D. M. Brown at the Laboratory for the Study of Hereditary and Metabolic Disorders, University of Utah School of Medicine.

In Table III, the electrophoretic composition of the whole serum and of the two fractions obtained with ammonium sulfate, as well as of the wash fluid, are presented. Fraction G.G.33.3 was relatively homogeneous, being 73 to 83 per cent γ-globulin. By simple calculation, it may be shown that Fraction G.G.33.3 contains from 70 to 82 per cent of the total γ-globulin present in whole serum. Most of the remainder is lost during the process of washing the precipitate.

Comparison of Chemical and Electrophoretic Values for γ-Globulin—In thirty-seven human sera (seven from normal subjects and thirty from patients with various diseases) the numerical value for γ-globulin as determined chemically was found to correlate reasonably well with the value for γ-globulin obtained by electrophoretic estimation (Fig. 1). The values are expressed as per cent of the total serum protein. The standard deviation of the chemical values from the electrophoretic values was 2.01 per cent, with a coefficient of variation of 10 per cent. The mean electrophoretic value for γ-globulin in the thirty-seven samples of sera was 17.9 per cent. A curve constructed to fit the points in Fig. 1 has the formula $y = 3.419 + 0.8498x$. The value of S^2 is 4.72.[3] The numerical values for γ-globulin (Fraction G.G.33.3) determined chemically are somewhat high in the normal range and somewhat low in the high range for corresponding values of γ-globulin determined electrophoretically.

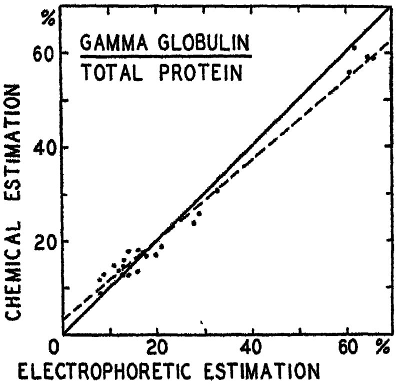

FIG. 1. The solid line would obtain if perfect correlation between chemical and electrophoretic methods for estimation of γ-globulin to total protein had occurred. The dotted line is derived from calculations to fit the observed points, assuming the electrophoretic estimations to be true values. Because of overlapping, the entire thirty-seven values cannot be represented graphically.

Solubility Studies—The precipitation of a γ-globulin fraction from undiluted serum with saturated ammonium sulfate may be criticized because of the likelihood of occlusion of other proteins in the precipitate and because of the greater tendency for lipide-rich β-globulin to adhere to the precipitate in undiluted serum compared with diluted serum (14). However, with dilution of serum prior to precipitation, the recovery becomes poor, particularly in those sera in which the γ-globulin fraction is not increased.

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[3] $S^2 = (\Sigma(y - y^0)^2)/(n - 2)$, where y = the observed value and y^0 = the calculated value.

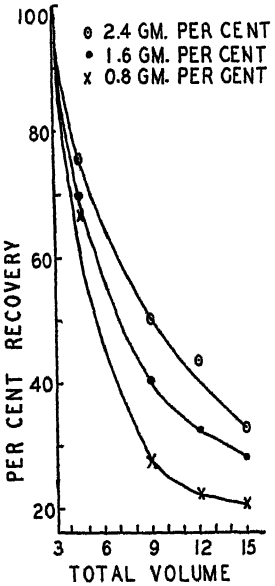

FIG. 2

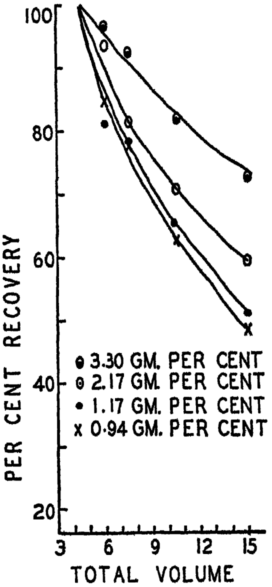

FIG. 3

FIG. 2. Quantity of protein recovered in the precipitate (as per cent of protein quantity introduced) (y) with increasing volumes of 33.3 per cent saturated ammonium sulfate solution (x) for three separate amounts of Fraction G.G.33.3.

FIG. 3. Effect of dilution upon the quantity of precipitate recovered for four specimens of whole serum in which increasing concentrations of Fraction G.G.33.3 were demonstrated by the usual method. Percentage recovery with increasing dilution is arbitrarily expressed as per cent of the protein precipitate obtained with a 4.5 ml. volume of salt solution.

A large quantity of washed and dialyzed Fraction G.G.33.3 was prepared from pooled normal human serum by the method outlined above. 1.0 ml. quantities of a given concentration of protein solution were diluted with increasing volumes of saline prior to addition of sufficient saturated ammonium sulfate to produce 33.3 per cent saturation. In each instance the precipitate was washed with 3.0 ml. of 33.3 per cent saturated ammonium sulfate. The total volumes of solution in contact with each protein concentration ranged from 4.5 ml. for undiluted protein solution (1 ml. of protein solution + 0.5 ml. of salt + 3.0 ml. of wash fluid) to 15 ml. (dilution with 7 ml. of NaCl). These procedures were carried out for protein concentrations of 2.40, 1.60, and 0.80 gm. per cent. The quantity of protein in the precipitate was measured and was expressed as per cent of the original protein concentration of the solution. As is seen in Fig. 2, the greater the total volume of solution in contact with protein, the less the amount of protein that may be expected to precipitate. Moreover at any given volume, the percentage recovery of the protein introduced becomes less with decreasing concentrations of protein in the original solutions. Similar results were obtained with a sample of Cohn's Fraction II (13). This fraction, however, was far more soluble in 33.3 per cent saturated ammonium sulfate than was ours.

In Fig. 3 is illustrated a similar study on normal sera and pathologic sera in which Fraction G.G.33.3 content was increased. Here the amount of precipitate present with the usual procedure (4.5 ml. of the total volume) is taken to represent 100 per cent yield and the precipitate recovered in diluted serum is expressed in relation to this amount in per cent. While the percentage recovery with dilution fell off steeply for sera with normal values for Fraction G.G.33.3, this was not the case for the pathologic sera, in which this fraction was increased. The similarity of these results with whole sera to those obtained in solutions of Fraction G.G.33.3 is evident

DISCUSSION

Because of the overlapping solubility characteristics of the various globulin fractions, there is no simple salting-out procedure which will permit quantitative recovery of a homogeneous γ-globulin. With the usual salting-out methods for globulin fractionation, the volume of salt solution added per ml. of serum is large. As is indicated by our solubility studies, such methods, if scaled to yield a homogeneous fraction of γ-globulin, will give a poor recovery when the γ-globulin content is normal or moderately increased and a relatively greater recovery when this constituent is considerably increased (2). Such methods adjusted to give recoveries of protein *numerically* equivalent to the electrophoretic content of γ-globulin in *normal* serum necessarily must involve the simultaneous precipitation of considerable amounts of other globulin fractions. The method that we have presented, while it does not yield a pure fraction of γ-globulin, permits sufficient recovery to detect relatively slight quantitative changes in the ranges in which this constituent is normal or moderately elevated as well as in the range in which it is greatly increased.

SUMMARY

A simple method is described for measuring the amount of protein precipitated from undiluted serum to which is added sufficient ammonium sulfate to produce 33.3 per cent saturation.

Electrophoretic studies indicate that this fraction consists mainly of γ-globulin and demonstrate a good numerical correlation between electrophoretic values for γ-globulin and chemical values in normal and abnormal sera.

Investigation of some solubility characteristics of this fraction reveals certain advantages in making the precipitation from undiluted rather than diluted serum.

Dr. G. R. Greenberg suggested this method to us. Dr. Emil L. Smith and Dr. M. M. Wintrobe gave helpful criticism.

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THE ACTION OF ENZYMES ON PARAMECIN*

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Paramecin, the "killer" substance of *Paramecium aurelia* (stock 51, variety 4), is easily inactivated under mild conditions, as is evidenced by the influence of the hydrogen ion concentrations on its stability (2). Paramecin is most stable at a hydrogen ion concentration of 8.0 to 8.5; nevertheless, 13 per cent of the activity is lost after an incubation of an hour at 30.0° at this pH. The heat of activation of the inactivation reaction, calculated from the reaction rates at 30° and 40°, was found to be 126,000 calories per mole at pH 7.0, a value typical for either an enzyme or a protein or both.

The analysis of paramecin by means of enzyme digestion experiments which seek to identify specific substances and linkages by specific enzymes might reveal some of the chemical nature of this compound. Methods of this kind have been invaluable in the analysis of the chemical nature of the chromosome by van Herwerden (3), Caspersson (4), Mazia (5), and Catcheside (6), and in studies by Brachet (7) on the rôle of nucleic acids during embryonic development. It could not be hoped that the application of the enzyme digestion technique would specifically identify paramecin as a chemical entity; however, valuable information might be obtained on the presence in the paramecin molecule of certain classes of compounds which are essential for its biological activity.

Methods and Results

Enzyme Preparations—The action of the following enzyme preparations on paramecin was investigated: (*a*) lysozyme in the form of its crystalline carbonate prepared by Alderton and Fevold (8); (*b*) a lysozyme preparation, MI35A, prepared by Meyer and Hahnel (9); (*c*) hyaluronidase, Preparation HD88-99, 35 viscosity reducing units per mg., prepared according to the procedure of Madinaveita (10) and obtained from Dr. Erwin Schwenk; (*d*) crystalline trypsin, obtained from Armour and Company; (*e*) papain, purified according to the procedure of Irving *et al.* (11); (*f*) pepsin (Merck and Company, Inc.); (*g*) crystalline chymotrypsin, obtained from Armour and Company; (*h*) crystalline ribonuclease (Dr. Kunitz); (*i*) crystalline desoxyribonuclease (Dr. McCarty); and (*j*) protein B, a proteolytic enzyme isolated from beef pancreas (12), obtained from Dr. Laskowski.

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Part of this study was presented in a symposium, "Plasmagenes, genes and characters in *Paramecium aurelia*," at the 114th meeting of the American Association for the Advancement of Science, Chicago, Illinois, December 26–31, 1947. A preliminary report is in press (1).

We want to express our deep appreciation to Dr. G. Alderton and Dr. H. L. Fevold of the Western Regional Research Laboratory, Dr. K. Meyer of Columbia University, Dr. Erwin Schwenk of the Schering Corporation, Dr. M. Kunitz and Dr. M. McCarty of The Rockefeller Institute for Medical Research, and to Dr. M. Laskowski of Marquette University for their more than generous gifts of several of the above enzyme preparations.

Method of Testing—A detailed description of the methods of cultivating *Paramecium aurelia* and of the procedure of testing the activity of paramecin extracts has been published (2). The action of the enzymes was determined by incubating the paramecin extract with the particular enzyme under investigation at 30.0°, while care was being taken in each case to adjust the substrate in such a manner that optimal activity for the enzymic action was assured. Samples were taken after temperature adjustment and at different time intervals up to 60 minutes. After dilution the samples were tested in the usual way for activity, by distributing 1 cc. of the dilution to ten depression slides containing sensitive animals (*Paramecium aurelia*, stock 31, variety 8). After incubation for 48 hours at 27° the dead and affected animals were counted. The actual counts were recalculated on a percentage basis. A control in phosphate buffer of pH 7.0 and a control in which the substrate had the same composition as the one used in the enzyme experiment, but in which the enzyme was replaced by a buffer solution, were included in each experiment. All enzymes were tested in the concentration of the final dilution for any action on sensitive paramecia. In no instance could any lethal effect or paramecin activity of the various enzymes be detected. All the data given are averages of at least duplicate determinations.

Lysozyme—Lysozyme activity has an optimum at pH 5.3 when the activity is measured viscosimetrically. At this hydrogen ion concentration paramecin is very rapidly inactivated. However, a second optimum can be demonstrated at pH 7.0 when lysozyme activity is measured by the increase in reducing groups in the substrate. The depolymerase action of lysozyme at this pH is only slightly less than at its optimum. The addition of sodium chloride in a concentration of 0.2 M is necessary to insure highest activity of the depolymerizing action of lysozyme, while the opening of glucosidic linkages does not seem to require halogen (9). It was decided therefore to investigate the action of lysozyme on paramecin in the presence of sodium chloride. It is clear from Table I that the two lysozyme preparations had no effect on the activity of paramecin. No significant difference is apparent between the inactivation in the absence and in the presence of the enzyme. It should be noted that paramecin is much more unstable in the presence of 0.2 M sodium chloride than when sodium chloride is omitted from the substrate.

TABLE I

Action of Lysozyme on Crude Paramecin Extracts

Samples of 0.05 cc. were taken at 30 and 60 minutes and diluted 1:500 before 1 cc. of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours. The buffer added to Control 2 was 0.4 M in NaCl. All of the lysozyme preparations were dissolved in phosphate buffer of pH 7.0, which contained 0.4 M NaCl. The final concentration of NaCl was 0.2 M in every experiment, except Control 1, which contained no salt.

	Composition of medium	Per cent activity		
		0 min.	30 min.	60 min.
Control 1	1.0 cc. paste 1.0 " buffer	100.0	72.3	45.2
" 2	1.0 " paste 1.0 " buffer, 0.2 M NaCl	100.0	47.8	20.4
" 3	1.0 " lysozyme, 200 γ per cc. 1.0 " buffer	0	0	0
Lysozyme MI35A, 1 γ per cc.	1.0 " paste 1.0 " lysozyme, 2 γ per cc.	100.0	45.9	21.2
Lysozyme MI35A, 10 γ per cc.	1.0 " paste 1.0 " lysozyme, 20 γ per cc.	100.0	44.8	19.3
Lysozyme MI35A, 100 γ per cc.	1.0 " paste 1.0 " lysozyme, 200 γ per cc.	100.0	46.2	22.0
Lysozyme W. R.,* 1 γ per cc.	1.0 " paste 1.0 " lysozyme, 2 γ per cc.	100.0	40.1	19.0
Lysozyme W. R.,* 10 γ per cc.	1.0 " paste 1.0 " lysozyme, 20 γ per cc.	100.0	43.5	18.0
Lysozyme W. R.,* 100 γ per cc.	1.0 " paste 1.0 " lysozyme, 200 γ per cc.	100.0	49.2	22.3

* Western Regional Research Laboratory.

Hyaluronidase—The presence of hyaluronidase in pathogenic bacteria (13–16) and spermatozoa (17–19) suggests that this enzyme plays a rôle in the processes of invasion by the depolymerization of the mucoid ground substance of connective tissue. Evidence has also been presented that hyaluronidase and the "spreading factor," which increases the permeability of the host tissue, are identical (20–22). Paramecin extracts were incubated with hyaluronidase in different concentrations. The results, which are reported in Table II, indicate that hyaluronidase is without any effect on the activity of paramecin.

Papain—Papain was purified according to the procedure of *Irving et al.* (11). The final precipitate was dried *in vacuo*. After it was equilibrated with the moisture of the laboratory atmosphere, it contained 12.79 per cent Kjeldahl N, and had about the same activity as the product obtained by the above workers. Hoover and Kokes (23) reported that the initial rate of digestion by papain has an optimum at pH 7. Although the optimum for final digestion of proteins is at pH 5, it was decided to test papain at a pH of 7, since there seems to be no essential difference in the attack of the enzyme on proteins. The enzyme was activated by incubating 25 mg. of the purified preparation in 25 cc. of phosphate buffer (pH 7.0) with an equal amount of cysteine for 1 hour at 30.0°. It can be seen from Table III that papain did not inactivate paramecin.

TABLE II

Action of Hyaluronidase on Crude Paramecin Extracts

The enzyme was dissolved in phosphate buffer, pH 7.0. Samples were taken at the designated time intervals and diluted 1:500 before testing. 1 cc. of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours.

	Composition of medium	Per cent activity			
		0 min.	20 min.	40 min.	60 min.
Control 1	1.0 cc. paste 1.0 " buffer, pH 7.0	100.0	79.7	65.5	42.0
" 2	1.0 " hyaluronidase, 20 mg. per cc.	0	0	0	0
Hyaluronidase, 10 mg. per cc.	1.0 cc. paste 1.0 " hyaluronidase, 20 mg. per cc.	100.0	76.0	63.7	45.9
Hyaluronidase, 1 mg. per cc.	1.0 cc. paste 1.0 " hyaluronidase, 2 mg. per cc.	100.0	77.0	63.6	45.0
Hyaluronidase, 0.1 mg. per cc.	1.0 cc. paste 1.0 " hyaluronidase, 0.2 mg. per cc.	100.0	81.0	62.8	41.5

Trypsin—Apparent inactivation to about 20 per cent of the original activity took place within the first 20 minutes when paramecin extracts were incubated with trypsin. Hereafter no further inactivation took place. This behavior suggested that paramecin formed an inactive complex with trypsin, similar to those formed by tobacco mosaic virus and trypsin, as reported by Stanley (24), Loring (25), and Kleczkowski (26). In order to test this, 4 cc. of a paramecin extract were incubated with 1 mg. of trypsin for 20 minutes at 30.0°. This suspension was then tested without dilution and in increasing dilutions (Table IV). Apparently trypsin inactivates paramecin by reversible complex formation. The complex dissociates easily upon dilution.

TABLE III

Action of Papain on Crude Extracts of Paramecin

Samples were taken at the designated time intervals and diluted 1:500 before testing. 1 cc. of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours.

	Composition of medium	Per cent activity			
		0 min.	20 min.	40 min.	60 min.
Control 1	1.0 cc. paste 1.0 " buffer, pH 7.0	100.0	76.0	60.0	47.0
" 2	1.0 " paste 1 cc. buffer, pH 7.0 1 mg. cysteine per cc.	100.0	97.0	96.0	94.0
Papain	1.0 cc. paste 1 cc. buffer, pH 7.0 Papain, 1 mg. per cc. Cysteine, 1 mg. per cc.	100.0	98.5	95.5	92.5

TABLE IV

Dissociation of Inactive Complex of Paramecin with Trypsin on Dilution

	Dilution	Nos. of sensitive *Paramecia* killed per cc. of diluted paste	Total activity
Control	1:10,000	61	610,000
Trypsin		96	96
	1:100	764	76,400
	1:1,000	127	127,000
	1:10,000	57	570,000
	1:100,000	5	500,000

Pepsin—Stanley (27) reported that tobacco mosaic virus was inactivated when incubated with pepsin under conditions favorable for optimal proteolytic activity of pepsin. The optimal pH for the proteolytic activity of pepsin is 2.0, a hydrogen ion concentration at which paramecin is instantaneously inactivated (1). The lowest hydrogen ion concentration at which different rates of inactivation could be determined with any degree of accuracy was that of pH 6. At this pH pepsin still exerts proteolytic activity (28). It was found (Fig. 1) that the presence of pepsin in the substrate caused a greater inactivation than that found in the control.

Chymotrypsin—Crystalline chymotrypsin was dissolved in a phosphate buffer of pH 8. The solution contained 2 mg. of chymotrypsin per cc.

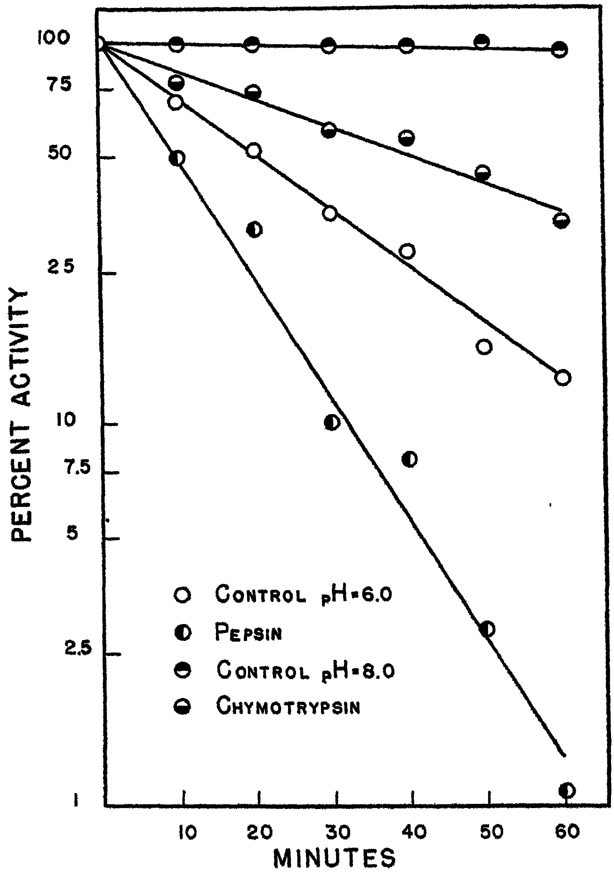

FIG. 1. Inactivation of paramecin by pepsin and crystalline chymotrypsin. Pepsin, 1 cc. of paramecium paste phosphate buffer (pH 6.0) was incubated with 1 cc. of a pepsin solution (Merck, U. S. P., granular, 2 mg. per cc. in phosphate buffer, pH 6.0) at 30°; chymotrypsin, 1 cc. of *Paramecium* paste in phosphate buffer (pH 8.0) was incubated with 1 cc. of a chymotrypsin solution (Armour and Company, crystalline, 2 mg. per cc. in phosphate buffer, pH 8.0) at 30°.

Incubation of a paramecium paste with this chymotrypsin solution resulted in a greater loss of activity than in the corresponding control (Fig. 1).

Ribonuclease—When a paramecium paste was incubated with varying concentrations of ribonuclease (RNase) in the presence of Mg^{++} ions (29), no inactivation of paramecin, other than the inactivation normally occurring at the pH of 7.4, was found to occur (Table V). It is also clear from Table V that Mg^{++} ions do not affect the inactivation of paramecin.

Desoxyribonuclease—The most revealing experiments were those conducted with desoxyribonuclease. It can be seen from Fig. 2 that paramecin is inactivated rapidly when incubated with varying amounts of desoxyribonuclease.

TABLE V

Incubation of Paramecin with Ribonuclease

The ribonuclease and $MgSO_4$ were dissolved in phosphate buffer, pH 7.4. Samples were taken at the specified time intervals and diluted 1:500 before testing. 1 cc. of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours.

	Composition of medium	Per cent activity			
		0 min.	20 min.	40 min.	60 min.
Control 1	4.0 cc. paste 1.0 " buffer, pH 7.4	100.0	88.1	76.5	65.5
" 2	4.0 " paste 0.5 " buffer, pH 7.4 0.5 " Mg^{++} solution, 0.25 M	100.0	84.5	70.5	64.5
" 3	4.0 " paste 0.5 " buffer, pH 7.4 0.5 " RNase, 200 γ per cc.	100.0	85.6	73.4	65.0
RNase, 20 γ per cc.	4.0 " paste 0.5 " RNase, 200 γ per cc. 0.5 " Mg^{++} solution, 0.25 M	100.0	81.0	74.0	65.5
" 2 " " "	4.0 " paste 0.5 " RNase, 20 γ per cc. 0.5 " Mg^{++} solution, 0.25 M	100.0	82.5	73.1	63.1
" 0.2 " " "	4.0 " paste 0.5 " RNase, 2 γ per cc. 0.5 " Mg^{++} solution, 0.25 M	100.0	84.1	71.9	63.9

Desoxyribonuclease is specifically activated by Mg^{++} and Mn^{++} ions in concentrations as low as 0.003 M. The activation by Mg^{++} ions is inhibited by citrate in a concentration of 0.01 M, while the Mn^{++} activation is not (30). Experiments were designed to test whether the inactivation of paramecin by desoxyribonuclease was prevented by omitting magnesium from the substrate and whether addition of citrate to the complete test solution would have any influence on the inactivation of paramecin by desoxyribonuclease in the presence of either magnesium or manganese ions. It is clear from Fig. 3 that magnesium in a concentration of 0.003 M is essential for the inactivation of paramecin by the enzyme. A slight inactivation is found if magnesium is omitted from the substrate. However, since no special precautions were taken to exclude all traces of magnesium from the solutions, this slight inactivation might be due to the presence of traces of this metal in the substrate. The addition of citrate prevents paramecin breakdown when magnesium ions are the activators of desoxyribonuclease (Table VI). When manganese ions are present in the substrate, addition of citrate is ineffective (Table VII).

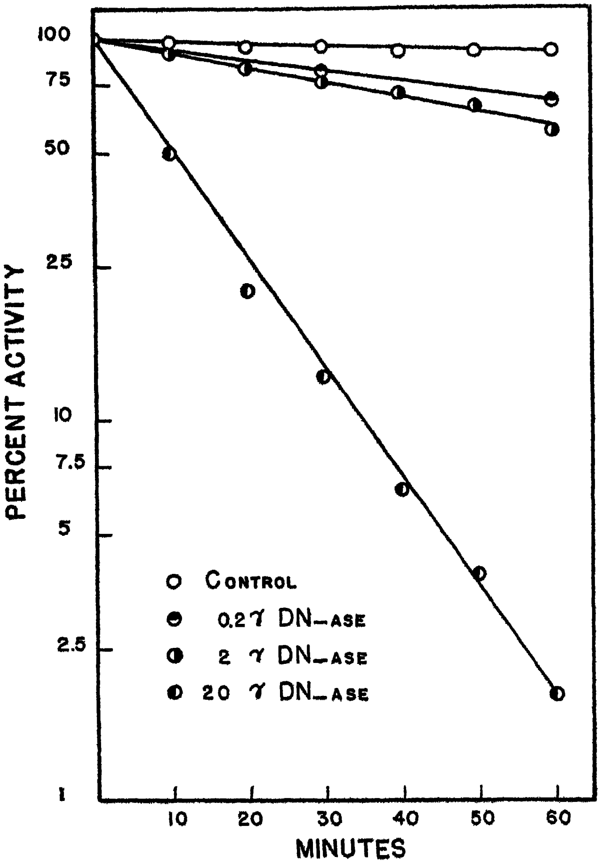

Fig. 2. Inactivation of paramecin by different concentrations of desoxyribonuclease. The paste of *Paramecium aurelia* was prepared by breaking the animals up in a 0.01 M phosphate buffer, pH 7.6, containing 0.25 per cent gelatin and 0.003 Mg^{++} ions. The desoxyribonuclease was also dissolved in this buffer, in such a concentration that the final concentration in the test was like that indicated in the figure.

Protein B—The outcome of these experiments can be interpreted to mean that desoxyribonucleic acid is an integral part of paramecin. However, Laskowski *et al.* (12, 31) have reported the isolation of a proteolytic enzyme,

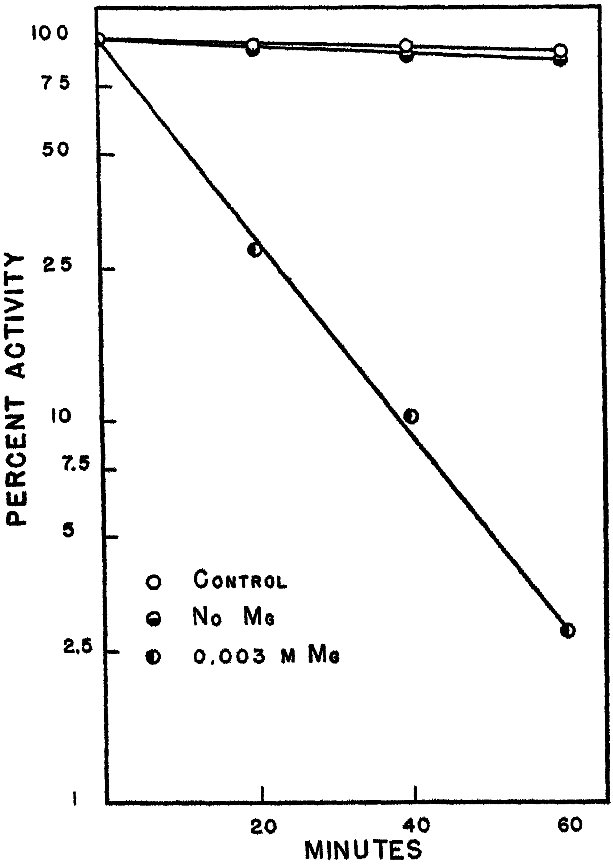

FIG. 3. The influence of Mg^{++} ions on the inactivation of paramecin by desoxyribonuclease. The composition of the medium in the different runs was as follows: ○, 3.0 cc. of paste made in phosphate buffer (pH 7.6, gelatin 0.25 per cent); 1.0 cc. of Mg^{++} solution, 0.015 M (pH 7.6, gelatin 0.25 per cent); 1.0 cc. of buffer (pH 7.6, gelatin 0.25 per cent). ◒, 3.0 cc. of paste made in phosphate buffer (pH 7.6, gelatin 0.25 per cent); 1.0 cc. of desoxyribonuclease in phosphate buffer (pH 7.6, gelatin 0.25 per cent, DNase, 100 γ per cc.); 1.0 cc. of buffer (pH 7.6, gelatin 0.25 per cent). ◐, 3.0 cc. of paste made in phosphate buffer (pH 7.6, gelatin 0.25 per cent); 1.0 cc. of desoxyribonuclease (pH 7.6, gelatin 0.25 per cent, DNase, 100 γ per cc.); 1.0 cc. of Mg^{++} solution, 0.015 M (pH 7.6, gelatin 0.25 per cent).

presumably of the chymotrypsin type, from desoxyribonuclease preparations, prepared according to the method of McCarty (30). It could therefore be possible that the observed inactivation of paramecin by desoxyribonuclease preparations was entirely due to contamination of desoxyribonuclease by a proteolytic enzyme. In order to test whether the observed inactivation of paramecin by desoxyribonuclease was indeed due to a specific action of the enzyme, the action of protein B, the proteolytic enzyme obtained from desoxyribonuclease preparations by Laskowski, was tested. It is evident from Table VIII that this enzyme, even when tested in concentrations 1000 times greater than those used in the desoxyribonuclease experiments, does not inactivate paramecin.

TABLE VI

Influence of Mg^{++} and Citrate on Inactivation of Paramecin by Desoxyribonuclease

Desoxyribonuclease, $MgSO_4$, and Na citrate were dissolved in phosphate buffer, pH 7.6. Samples were taken at the specified time intervals and diluted 1:500 before testing. 1 cc. of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours.

	Composition of medium	Per cent activity			
		0 min.	20 min.	40 min.	60 min.
Control 1	3.0 cc. paste 2.0 " buffer, pH 7.6	100.0	93.7	91.7	90.0
" 2	3.0 " paste 1.5 " buffer, pH 7.6 0.5 " Mg^{++} solution, 0.03 M	100.0	94.3	92.4	89.4
" 3	3.0 " paste 1.5 " buffer, pH 7.6 0.5 " citrate, 0.1 M	100.0	92.5	92.0	88.8
" 4	3.0 " paste 1.0 " DNase, 100 γ per cc. 1.0 " buffer, pH 7.6	100.0	96.0	92.0	91.0
DNase + Mg^{++}	3.0 " paste 1.0 " DNase, 100 γ per cc. 0.5 " Mg^{++} solution, 0.03 M 0.5 " buffer, pH 7.6	100.0	23.4	6.7	3.3
" + " + citrate	3.0 " paste 1.0 " DNase, 100 γ per cc. 0.5 " Mg^{++} solution, 0.03 M 0.5 " citrate, 0.1 M	100.0	94.8	91.8	87.5

DISCUSSION

It can be tentatively concluded from the experiments reported above that paramecin is a desoxyribonucleoprotein. Inactivation of the killer principle by pepsin and chymotrypsin indicates that a protein is an integral part of the paramecin essential for its activity. The specific inactivation by

TABLE VII

Influence of Mn^{++} and Citrate on Inactivation of Paramecin by Desoxyribonuclease

Desoxyribonuclease, $MnSO_4$, and Na citrate were dissolved in phosphate buffer, pH 7.6. Samples were taken at the specified time intervals and diluted 1:500 before testing. 1 cc. of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours.

	Composition of medium	Per cent activity			
		0 min.	20 min.	40 min.	60 min.
Control 1	3.0 cc. paste 2.0 “ buffer, pH 7.6	100.0	97.5	91.0	91.0
“ 2	3.0 “ paste 1.5 “ buffer, pH 7.6 0.5 “ Mn^{++} solution, 0.03 M	100.0	95.6	93.7	91.7
“ 3	3.0 “ paste 1.5 “ buffer, pH 7.6 0.5 “ citrate, 0.1 M	100.0	94.5	92.5	90.8
“ 4	3.0 “ paste 1.0 “ DNase, 100 γ per cc. 1.0 “ buffer, pH 7.6	100.0	94.2	94.2	92.0
DNase + Mn^{++}	3.0 “ paste 1.0 “ DNase, 100 γ per cc. 0.5 “ Mn^{++} solution, 0.03 M 0.5 “ buffer, pH 7.6	100.0	31.0	5.7	3.2
“ + “ + citrate	3.0 “ paste 1.0 “ DNase, 100 γ per cc. 0.5 “ Mn^{++} solution, 0.03 M 0.5 “ citrate, 0.1 M	100.0	31.6	6.1	2.5

TABLE VIII

Action of Protein B on Crude Extracts of Paramecin

Protein B was dissolved in phosphate buffer, pH 7.8. Samples were taken at the specified time intervals and diluted 1:500 before testing. 1 cc. of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours.

	Per cent activity			
	0 min.	20 min.	40 min.	60 min.
Control, pH 7.8	100.0	98.0	92.0	92.0
Protein B, 20 mg. per cc.	100.0	97.2	94.5	91.0
“ “ 10 “ “ “	100.0	97.0	88.5	88.5
“ “ 1 “ “ “	100.0	90.7	87.5	85.5

desoxyribonuclease suggests that a desoxyribonucleic acid is also required for the activity. It is fully realized that a definite conclusion as to the chemical nature of paramecin can be reached only after this compound has been isolated in the pure and active state.

Nevertheless, several important facts emerge from this study. The inactivation of paramecin by both pepsin and chymotrypsin and the fact that neither papain nor trypsin influences the activity of paramecin (the apparent inactivation of paramecin by trypsin is not due to proteolytic action of this enzyme) indicate that basic amino acids such as arginine and lysine are probably not essential for the activity of paramecin, for both pepsin and chymotrypsin require as groups in the side chain of the substrate phenylalanine or tyrosine residues, while the requisite groups for trypsin are those of the arginine and lysine residues (32).

The available evidence seems to indicate the presence of a desoxyribonucleoprotein in the cytoplasm of *Paramecium aurelia.* Bensley (33), Hoerr (34), and Lazarow (35) reported the isolation of a cytoplasmic component, plasmosin, of the desoxyribonucleoprotein type. Their view-point is not shared by Mirsky and Pollister (36), who maintain that plasmosin is located largely, if not entirely, in the nucleus of the cell, it being a constituent of chromatin. It seems to be generally agreed that nucleic acids of the desoxyribose type are never regular constituents of the cytoplasm, and any desoxyribonucleic acid found in cytoplasmic preparations is thought to be present there because the methods used for the isolation tend to dissolve the content of the nucleus, thereby liberating desoxyribonucleic acid of nuclear origin (37, 38). However, Sparrow and Hammond (39) published evidence, based on the Feulgen reaction and on ultraviolet adsorption data, that bodies containing desoxyribonucleic acid are present in the cytoplasm of meiotic stages of micro sporocytes from eight genera of plants. Furthermore, the presence of very small quantities of desoxyribonucleic acid in the cytoplasm has never been completely excluded (36–38, 40). It then would not seem unlikely that, in view of the extreme biological activity of paramecin, only minute traces of this compound were present (41).

The importance of desoxyribonucleic acid as a cellular determinant is well established through the work of Avery *et al.* and McCarty (42–44) on the transforming principle of *Pneumococcus.* Amounts as small as 0.001 γ will transform non-encapsulated R variants of *Pneumococci* into encapsulated S variants. Boivin and associates (45–47) have presented evidence that a desoxyribonucleic acid acts as a transforming principle in *Escherichia coli,* similarly changing the antigenic type. In *Paramecium aurelia* a desoxyribonucleic acid is an integral part of a substance responsible for a specific killing action. Preer (48) recently reported that Feulgen-positive bodies, having the physiological properties of the cytoplasmic factor κ, are present in the cytoplasm of killer animals. Sonneborn (49) has presented evidence that sensitive animals, homozygous for the gene K, can be transformed into true killer animals by exposure to large concentrations of cytoplasmic material obtained from killer animals. A suggestive working hypothesis could postulate that paramecin is closely related to the cytoplasmic factor which then in *Paramecium aurelia* would have a similar function as the transforming principle of *Pneumococcus* and *Escherichia coli*. The apparent difference between the two cases is that transformation in *Paramecium aurelia* is brought about by a desoxyribonucleoprotein, while desoxyribonucleic acid alone is capable to act as a transforming agent for *Pneumococcus* and *E. coli*. However, it might be possible that in the latter case desoxyribonucleic acid acts as a prosthetic group of the transforming principle, *Pneumococci* and *E. coli* being able to synthesize the protein moiety. *Paramecium aurelia*, unable to synthesize the protein part, would require the whole unsplit desoxyribonucleoprotein.

SUMMARY

Paramecin is inactivated by pepsin, chymotrypsin, and desoxyribonuclease, indicating the presence of a protein and a desoxyribonucleic acid in the compound. Both parts are essential for the killing action of paramecin. The inactivation of paramecin by desoxyribonuclease is specifically activated by magnesium and manganese ions. The inactivation of paramecin by desoxyribonuclease in the presence of magnesium ions is inhibited by citrate, in contrast to the inactivation by this enzyme in the presence of manganese ions, which is unaffected by the addition of citrate to the substrate. The inactivation by desoxyribonuclease is not due to the presence of a contaminating proteolytic enzyme. This enzyme, protein B, does not inactivate paramecin in concentrations 1000 times greater than that of desoxyribonuclease. Lysozyme, hyaluronidase, papain, and ribonuclease did not inactivate paramecin. Trypsin apparently formed an inactive complex with paramecin, which dissociated on dilution. The implications of these findings in relation to the cytoplasmic factor κ are discussed.

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THE PREPARATION AND PROPERTIES OF A LYSOPHOSPHOLIPASE FROM PENICILLIUM NOTATUM*

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In a previous communication (1) the name *phospholipase* was used to describe the enzyme in various venoms which converts lecithins and cephalins to lysolecithins and lysocephalins, with liberation of unsaturated fatty acids. The present work describes a powerful enzyme occurring in *Penicillium notatum*, which acts upon lysophospholipides to produce glycerylphosphorylcholine (or glycerylphosphorylethanolamine), with liberation of saturated fatty acids. This enzyme will be called *lysophospholipase*. Phospholipase and lysophospholipase are terms synonymous with lecithinase A and lecithinase B (2), respectively.

Investigation of the enzymatic degradation of the phospholipides has met with scant success when animal tissues have served as sources of the various enzymes concerned. This has not been due to absence of such enzymes from the tissues, for their existence has been amply proved through the study of autolytic processes (3, 4). In order to improve our understanding of the phospholipide-hydrolyzing enzymes, it has been considered advisable to look for sources of the individual enzymes which would make possible the study of each step in the degradation process. The classic example among such sources is, of course, the extremely active phospholipase of venoms, previously mentioned. In 1933 Contardi and Ercoli (5) described a lysophospholipase obtained from rice bran, rice embryos, and *Aspergillus oryzae*, whose activity was, however, quite low. *Penicillium notatum* has now been found to be an excellent source of this enzyme. Methods have been devised for the quantitative assessment of the reaction and for the determination of lysophospholipase activity. Certain properties of the enzyme have also been examined. The investigation has been discontinued.

EXPERIMENTAL

Enzyme—*Penicillium notatum*[1] was surface-cultured in 500 ml. Erlenmeyer flasks, on a corn steep, lactose-containing medium (6), for 5 to 8 days

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[1] The *Penicillium* strain used was kindly supplied by Dr. G. B. Reed.

at 25°. The mat of abundantly sporulating mold was then removed from the culture medium, washed with water, and dried superficially by pressing gently between filter papers. After crumbling the mat between the fingers, drying was completed *in vacuo* over anhydrous calcium sulfate (Drierite). Each culture yielded more than 1 gm. of dry mold, which was stored at 4° until required.

Extracts of this material were prepared by pulverizing 5 gm. portions, mixed with a little sand, adding 50 ml. of distilled water plus 5 drops of toluene, and autolyzing for 24 hours at 20–25°. After filtration and washing with suction, the combined filtrates (60 ml.) were dialyzed for 24 hours at 4°, with inside stirring, against 20 liters of running distilled water. After dialysis, one of two procedures was followed: (1) The extract was diluted to 75 ml. and stored at 4° under toluene; (2) the solution, in the dialysis sac, was suspended in glycerol and concentrated to about 14 ml., after which it was removed from the sac, diluted to 75 ml. with glycerol and stored at 4°.

Aqueous extracts lost about 80 per cent of their activity when stored at 4° for 2½ months. During the same period of time glycerol preparations remained fully active.[2] The nitrogen content of the dialyzed aqueous extracts varied from 0.280 to 0.380 mg. per ml.

Substrate—Lysophospholipides were prepared from egg yolks by a simplification of King's procedure (7), in which troublesome concentration of extracts *in vacuo* was eliminated. After formation of the lysophospholipides by incubation of 60 yolks with moccasin venom (*Agkistrodon piscivorus*), the reaction mixture (3 liters) was extracted with acetone (6 liters) at 50°, and filtered rapidly while still hot. The residue was reextracted twice with acetone (2 liters) and the combined filtrates cooled to 20°. To this were then added 90 ml. of saturated aqueous cadmium chloride solution, and the mixture was cooled to 0°. The copious precipitate was filtered off rapidly and extracted 5 times in a Waring blendor with 300 ml. portions of cold acetone. It was then dried *in vacuo*, following which lysophospholipides were isolated from the cadmium chloride complex by the usual methods. Yield, 35 gm.; P 5.70 per cent, N 2.72 per cent, NH_2-N (8) 0.552 per cent, iodine value of constituent fat acids 4.

Lysophospholipide sols were prepared as required, usually at concentrations of 1 or 0.5 per cent, by shaking with water or buffer solution at 40–50°. Such sols were stable at 30°, but precipitated slightly at 25° and heavily at 4°. They were chemically stable, at pH 4.0, for a period of several weeks and after storage at 4° were readily reformed by gentle heating.

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[2] As a result of the considerable stability of lysophospholipase in solution, extensive efforts to purify the enzyme, or to produce dry preparations, were not made. It could be precipitated partially from solution by saturated ammonium sulfate, by acetone, and by acid (pH 3.0), but on the basis of nitrogen content, none of these treatments increased the activity appreciably.

Reaction Mixture—Unless otherwise specified, the reaction mixture contained 1 ml. of 1 per cent lysophospholipides and 0.04 ml. of enzyme, in a total volume of 2.2 ml. All components of the final mixture were prepared in veronal-acetate buffer, pH 4.0. For the reaction, all components except enzyme were mixed in glass-stoppered test-tubes, and equilibrated for 20 minutes in a water bath maintained at 30°. The enzyme was added, and after 5 minutes a 1 ml. aliquot was removed into precipitation tubes as described below.

Under these conditions, the originally clear solution became quite turbid, owing to liberation of insoluble fatty acids, and if allowed to stand, formed a stiff gel. Degradation of the lysophospholipides varied from 15 to 50 per cent after 5 minutes, depending upon the age of the lysophospholipase used.

Quantitative Determination of Reaction—Preliminary experiments indicated that nephelometric or viscosimetric methods could not be applied as quantitative assessments of the reaction. Efforts were made, therefore, to separate the substrate from one or both of the reaction products. It was found that a satisfactorily complete separation of lysophospholipides and fatty acids from glycerylphosphorylcholine[3] could be made by the colloidal iron-magnesium sulfate method of Folch and Van Slyke (9). Lysophospholipides in the precipitate were then determined by analysis for phosphorus.

Details of the procedure were as follows: 1 ml. aliquots of the reaction mixture were added to 12 ml. conical centrifuge tubes containing 2.8 ml. of veronal-acetate buffer, pH 9.2, plus 4.2 ml. of water, and stirred immediately. The alkaline pH of this mixture effectively stopped the reaction. To the tubes were then added with stirring 0.5 ml. of colloidal iron (Fe_2O_3, 5 per cent dialyzed) and 0.5 ml. of half saturated magnesium sulfate solution. Addition of these acid reagents lowered the pH of the mixture to about 6.5. The tubes were centrifuged for 3 minutes, the supernatant solution containing the GPC, decanted, and the precipitates washed with 10 ml. of 0.05 saturated magnesium sulfate solution. To the centrifuged, washed precipitates were then added 3 ml. of water, in which the precipitates were suspended by thorough stirring, and transferred by aspiration to micro-Kjeldahl digestion flasks. The centrifuge tubes were washed carefully with two 1 ml. portions of sulfuric acid (86 per cent by volume), and the washings transferred to the digestion flasks. Digestion was carried out by adding 0.200 gm. of potassium sulfate-copper sulfate mixture (9:1) and boiling for 1 hour. An acid-washed Hengar granule effectively overcame the tendency to bump. After digestion, any precipitate present was filtered off, and phosphorus was determined colorimetrically (10). The presence of iron, magnesium, copper, and potassium ions was without effect on the determination.

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[3] The abbreviation GPC will be used to denote the mixed esters, glycerylphosphorylcholine and glycerylphosphorylethanolamine.

Identical amounts of lysophospholipides carried through the entire precipitation and digestion procedures showed a maximum variation in the analyses of ±1 per cent. The absolute amounts of phosphorus recovered in the precipitate, however, varied from 97 to 98 per cent of those found by direct analysis of lysophospholipides. There was, thus, a constant error of 3 per cent in the precipitation method. No correction was made for this loss.

In order to test the completeness with which lysophospholipide phosphorus and GPC phosphorus could be separated, varying amounts of the latter[4] were added to a constant amount of lysophospholipides, following which the precipitation procedure was applied. As shown in Table I, the separation was complete.

TABLE I

Separation of Lysophospholipide Phosphorus from GPC Phosphorus

P found*	GPC P added	GPC P as per cent of total P	Recovery of lysophospholipide P
mg.	*mg.*		*per cent*
0.270	0		97.5
0.272	0.0106	3.7	98.2
0.272	0.0212	7.1	98.2
0.270	0.0530	16.0	97.5
0.270	0.106	27.7	97.5
0.270	0.212	43.4	97.5

* Lysophospholipide P by direct analysis (without precipitation by iron-magnesium mixture) = 0.277 mg. All values are averages of duplicate samples.

The extent of the enzymatic reaction was calculated by comparison with the precipitated lysophospholipide phosphorus from a control tube. Since, in experimental tubes, the enzyme itself contained a small amount of precipitable phosphorus, an appropriate correction was made.

Under the conditions prescribed, addition of iron-magnesium to the precipitation tubes containing alkaline reaction mixture could be delayed 15 minutes, and after this addition the tubes could stand for at least 60 minutes before transfer of their contents to digestion flasks. In this way, it was possible to make twenty or more determinations in a single experiment.

Inorganic phosphate, if present, appeared in the precipitate with lysophospholipides. Glycerophosphate was partially precipitated. Thus, the method was valid only in the virtual absence of enzymes liberating choline or inorganic phosphate, or both, from GPC. It was possible to show that the enzyme preparation used liberated appreciable amounts of inorganic phosphate in the reaction mixture, at pH 4.0, only when present in high concentration (10 to 100 times the concentration ordinarily used) and over a prolonged time (4 to 8 hours). Similarly, in mixtures containing 5 times the usual concentration of enzyme, incubated for 60 minutes, no free choline was determinable by the sensitive reineckate method. Under the conditions specified, therefore, the only reaction occurring to a significant extent was the conversion of lysophospholipides to GPC and fatty acids.

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[4] A sample of pure racemic α-glycerylphosphorylcholine was kindly supplied by Dr. Erich Baer.

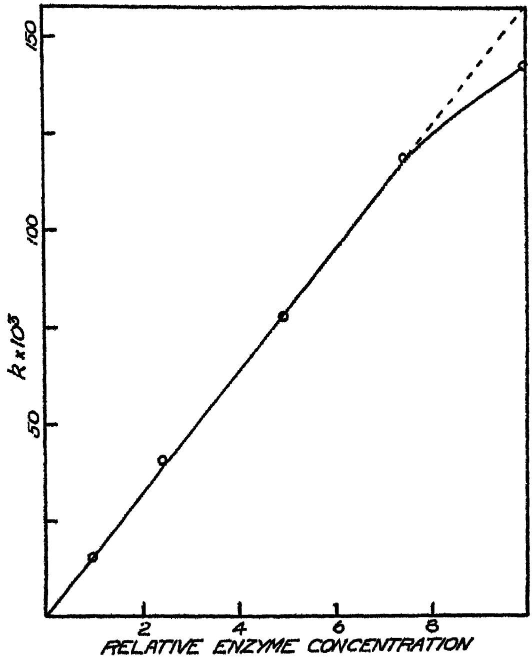

FIG. 1. Direct proportionality between first order constants (k) and lysophospholipase concentration, during the initial stages of reaction. The first point on the curve represents 7 per cent hydrolysis, the last point 53 per cent. Reaction time, 5 minutes.

Measurement of Activity—During the initial stages of the reaction the decrease in lysophospholipide phosphorus, in a given time, was not directly proportional to enzyme concentration. Under certain conditions, however, a first order reaction was simulated, the appropriate constant (k) being proportional to enzyme concentration over a considerable range (Fig. 1).

These conditions were (1) a reaction temperature of 30° (2) a reaction time of 15 minutes or less, (3) a per cent reaction less than 40.

Substrate Specificity—Repeated attempts were made to secure a reaction between lysophospholipase and lecithins or cephalins, all of them unsuccessful. The following phospholipide preparations were used: egg yolk and brain[5] lecithins purified according to Pangborn (11), petroleum ether-soluble egg yolk phospholipides (lecithins and cephalins), and the acetone-insoluble fraction of a commercial soy bean lecithin. With quantities of enzyme which extensively decomposed lysophospholipides (60 to 70 per cent) within 10 minutes, no action on lecithins was observed even after 1 hour.

Since the lysophospholipides in routine use as substrate for the enzyme contained 20.3 per cent of their total nitrogen as amino nitrogen, it was of interest to discover whether lysocephalins were reactive. In the absence of a method for obtaining pure lysocephalins, an indirect approach to the problem was made. 192 mg. of lysophospholipides (containing 1.11 mg. of amino nitrogen) were incubated for 1 hour with excess enzyme, at which time the reaction was 84 per cent complete. After separation of the residual lysophospholipides by the usual method, the GPC-containing solution was dried *in vacuo*, and analyzed for ethanolamine nitrogen (12). 0.573 mg. of ethanolamine nitrogen was found, corresponding to 52 per cent of the total amino nitrogen available for reaction. Lysophospholipase, therefore, acted upon lysocephalins as well as upon lysolecithins. Serine nitrogen (12) was not present in the substrate, and the enzyme itself contained neither ethanolamine nor serine nitrogen.

pH Optimum—The activity of lysophospholipase was determined at hydrogen ion concentrations varying from pH 2.88 to 6.43. The veronal-acetate buffer used exhibits constant ionic strength over this range (13). Sufficient enzyme was added to produce 31 per cent reaction at the most favorable hydrogen ion concentration. From the results (Fig. 2) it is seen that the optimum acidity for the reaction was rather sharply defined in the pH range 3.8 to 4.4. Contardi and Ercoli (5) found an optimum at pH 3.5 for the corresponding enzyme from *Aspergillus oryzae*.

Heat Stability—Fig. 3 contains a group of curves illustrating the resistance of lysophospholipase to various temperatures and hydrogen ion concentrations. In establishing these curves, solutions containing 0.6 ml. of enzyme extract per ml., at varying hydrogen ion concentrations, were heated at a given temperature (41°, 50°, or 61°) for a given time (15 or 30 minutes). The solutions were then cooled quickly, and their activity measured and compared with untreated enzyme. It is evident from the figure that the lysophospholipase exhibited a maximum heat stability near pH 4.5 and was rapidly inactivated at a pH greater than 7.0, at temperatures of 41° or higher.

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[5] The brain lecithin was a preparation kindly made available by Dr. R. G. Sinclair.

Activation and Inhibition—In general, lysophospholipase was resistant to most of the common enzyme inhibitors, and no activator was discovered.

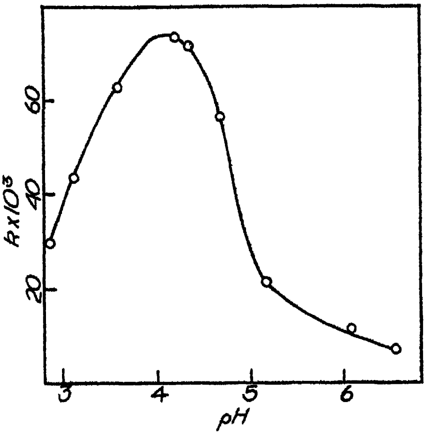

FIG. 2. pH-activity curve for lysophospholipase

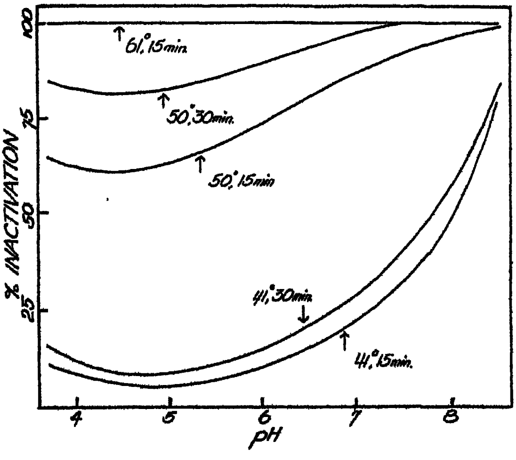

FIG. 3. Stability of lysophospholipase to heat and hydrogen ion concentration. Per cent inactivation = 100 (1 − (k (treated)/k (control))).

Cyanide was the most effective inhibitor among those tested (Table II), a 0.01 M concentration suppressing the reaction almost completely. Silver and copper ions were less effective. Calcium, magnesium, and cobalt ions did not inhibit the reaction, nor did sodium azide, cysteine, hydrogen sulfide, or hydrogen peroxide. The enzyme solution was slowly inactivated, presumably by surface denaturation, when air or nitrogen was bubbled through it.

As products of the reaction, GPC and fatty acids might be considered as inhibitors, since the reaction in the presence of the usual concentration of enzyme proceeded only to 60 to 70 per cent completion. Palmitic and linoleic acids (the latter a liquid acid not actually occurring in the substrate), added to the reaction mixture in amounts approximating their maximum concentration in the substrate, slightly inhibited the reaction. GPC, on the other hand, exerted no effect. Quite possibly the reaction was effectively suppressed before a chemical equilibrium was reached, as a result of the gel formation which occurred after some 60 per cent of the lysophospholipides were decomposed.

TABLE II

*Inhibition of Lysophospholipase**

Inhibitor	Concentration	Inhibition	Inhibitor	Concentration	Inhibition
	mole per l.	*per cent*		*mole per l.*	*per cent*
Palmitic acid	0.0094	18	$AgNO_3$	0.01	48
	0.00094	4		0.002	13
Linoleic acid	0.0089	11		0.001	8
	0.00089	6		0.0001	4
GPC	0.0097	2	KCN	0.01	97
	0.00097	0		0.001	87
$CuSO_4$	0.05	51		0.0002	48
	0.005	36		0.0001	12
	0.0001	21		0.00002	0

* Copper, silver, and cyanide ions were incubated with lysophospholipase for 30 minutes at 30° before addition to the substrate. During this time their concentration was 11 times that indicated in the table.

Francioli (14) reported the complete inhibition by physostigmine chloride of a comparable enzyme found in wasp venom. In our experiments physostigmine sulfate did not inhibit in a concentration as high as 1.0 mg. per ml., which was some 50 times greater than the concentration employed by Francioli. Since neither physostigmine chloride nor wasp venom was available to us for direct test, no explanation of this discrepancy in results can be advanced.

DISCUSSION

The name lysophospholipase implies a certain specificity of the enzyme for lysophospholipides. Contardi and Ercoli (5) and Francioli (14), after examining the corresponding enzyme derived from rice bran and embryos, *Aspergillus*, and wasp venom, concluded that phospholipides also served as substrate. The former workers, however, did not demonstrate in their preparations the absence of a true phospholipase. The results of Francioli, on the other hand, were based upon evidence acquired through the use of physostigmine as a lysophospholipase inhibitor. Our preparations, which were many times more active than those hitherto described, were completely inactive with respect to lecithins and cephalins, nor was it possible to demonstrate any inhibition of the reaction by physostigmine. Lysophospholipase from *Penicillium* may be regarded, therefore, as being specific for lysophospholipides.

Failure of *Penicillium* extracts to liberate inorganic phosphate from lysophospholipides or GPC was due not to lack of the acid phosphatase abundantly present in molds, but to a deficiency of GPCase, without which glycerophosphate could not be formed. Extracts of *Aspergillus oryzae*, prepared in the laboratory or obtained commercially,[6] contained considerable GPCase.

The lysophospholipase preparation used was relatively pure, *i.e.*, free of dialyzable substances, and low in nitrogen content. Each reaction tube, as ordinarily prepared, contained only 0.010 to 0.016 mg. of nitrogen added as enzyme. Efforts directed towards further purification of lysophospholipase should be rewarding.

SUMMARY

Lysophospholipase, a highly active enzyme specific in its action for lysophospholipides, was prepared from *Penicillium notatum*. The products of the reaction were saturated fatty acids and glycerylphosphorylcholine or glycerylphosphorylethanolamine (GPC).

Quantitative methods for determining the extent of the reaction were based upon the separation of residual lysophospholipides and liberated fatty acids from GPC by precipitation of the former with colloidal iron-magnesium sulfate mixture.

Lysophospholipase activity was determined, under prescribed conditions, by evaluation of the first order reaction constant.

The enzyme was readily inactivated by heat at a slightly alkaline reaction, by cyanide, and, less readily, by heavy metal ions. No activator was discovered. Optimum activity was at pH 4.0.

Grateful acknowledgment is made to Miss Katherine Justus for many of the analyses, and to Miss Eve Minovitch for the determinations of ethanolamine and serine nitrogen.

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[6] By courtesy of Mr. F. F. Taylor, Takamine Laboratory, Inc., Clifton, New Jersey.

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DETERMINATION OF PROTEIN-BOUND IODINE*

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The growing interest in chemical evaluation of thyroid function by use of the plasma level of protein-bound iodine (PI) is attested by the increasing numbers of reports in which this criterion is employed, both chemically and experimentally. However, the specific techniques available for such a determination do not yet seem sufficiently reliable. The purpose of this communication is to describe a procedure evolved in this laboratory which is consistent and sensitive in our hands.

The complete determination of plasma PI involves four distinct steps: (1) precipitation and washing of the plasma proteins; (2) digestion of the protein, leaving the PI in inorganic form; (3) distillation of the inorganic iodide; (4) actual determination of the inorganic iodide. The shortened technique of Salter (4), in which a dry ashing with NaOH and Na_2CO_3 was used to eliminate the need for distillation, has not proved satisfactory in our experience, and no other short cuts have been proposed.

In 1940, Chaney (1) described an all-glass still for use with a chromic-sulfuric acid digestion mixture; iodide catalysis of the reduction of ceric ions by arsenious acid was employed for the ultimate colorimetric determination of iodine. Although other workers have adopted the Chaney still (7), the highly sensitive ceric-arsenious catalysis method was generally avoided until Taurog and Chaikoff reported an entire procedure for plasma iodine (8). The iodide catalysis reaction had been extensively studied in 1937 by Sandell and Kolthoff (5), who demonstrated a marked enhancement of the iodide effect by the presence of a relatively high concentration of chloride. We have found that the use of chloride in optimum concentration improves the sensitivity 30 to 80 per cent with no sacrifice in reproducibility. None of the recently reported techniques has taken advantage of this appreciable increase in sensitivity.

EXPERIMENTAL

Reagents—

Distilled water. Although in the past it has been widely reported necessary to take the extraordinary precautions of freshly distilling the water from alkali, we have found it quite satisfactory merely to redistil once

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* Aided by a grant from the United States Public Health Service.

distilled water in an all-glass still. Since this is a low iodine region, the deficiency of iodine in the water may facilitate this. Furthermore, water so prepared and stored in a stoppered Pyrex flask for as long as 2 months at summer temperatures did not show any change from its extremely low iodine content or any effect on the recovery of iodide quantitatively added to it. All other reagents are made up in the double distilled water.

Somogyi precipitating reagents (*6*). The acid zinc sulfate solution contains 12.5 gm. of $ZnSO_4 \cdot 7H_2O$ and 125 ml. of 0.25 N H_2SO_4 per liter. NaOH is made up to 30 gm. per liter, and the two solutions are balanced until 50 ml. of the acid zinc sulfate require between 6.7 and 6.8 ml. of the alkali to show a permanent pink to phenolphthalein. A blank must be run on these reagents, carried through the washing procedures to be described.

Sulfuric acid solutions. 70 per cent by weight. 780 ml. of concentrated sulfuric acid, special, As- and N-free, are slowly added, with cooling, to 600 ml. of water in a 2 liter Pyrex flask. Thorough mixing should then be carried out.

1.0 N. 28 ml. of concentrated acid are carefully added to 900 ml. of water, and the solution cooled and made to 1 liter.

Chromic oxide. 600 gm. are dissolved in water, and made to 1 liter. This material has often been found to be contaminated; we have tested several batches of technical grade CrO_3 and found some to be very low in iodine. These particular samples are far less expensive than the high purity material, which is equally apt to be contaminated.

Sodium sulfite. A 10 per cent solution is used, which is made up fresh for each series of distillations by dissolving 2 gm. in 20 ml. of water.

Phosphorous acid, 50 per cent. 250 gm. are dissolved in about 200 ml. of water and made to 500 ml. If necessary the solution can be freed of iodine by boiling for ½ hour (with frequent addition of water); when cool, it is made to 500 ml. We have used the reagent from Fisher without boiling.

Arsenious acid. 3.71 gm. of As_2O_3 are dissolved in 50 ml. of N NaOH with stirring. 200 ml. of water are added and the solution neutralized with H_2SO_4 (requiring about 2.5 ml. of the 70 per cent solution). Then 54 ml. of the 70 per cent H_2SO_4 are added, and the solution made to 500 ml. 3.125 gm. of iodide-free NaCl are dissolved in the 500 ml. of reagent to avoid the need of another solution.

Ceric sulfate. 12 gm. of ceric ammonium sulfate (G. Frederick Smith) are stirred into 500 ml. of 3.5 N H_2SO_4. This will be turbid at first, clearing up within ½ hour upon occasional stirring.

Iodide standards. Pure NaI is carefully dried in a desiccator; 118.1 mg. are then dissolved and diluted to 1 liter. This stock solution contains 100 γ of I per ml., and must be appropriately diluted to yield the 0.005 to 0.100 γ of I desired as standards.

Special Apparatus—The Riggs modification of the Chaney still was employed as described by Talbot *et al.* ((7) p. 481) except that a 250 ml. flask was substituted for the 500 ml. flask. All grease was cleaned from both stop-cocks, and water was used as the only lubricant. Care must be taken to prevent freezing of the stop-cocks due to drying.

Procedure

This section falls logically into the four categories mentioned earlier.

1. Protein Precipitation and Washing—2 ml. of oxalated or heparinized plasma are precipitated by Somogyi's zinc sulfate reagent in a 50 ml. round bottom centrifuge tube. After a 10 minute period of centrifuging, the supernatant is poured off, and the precipitate washed free of inorganic iodide by four successive washings each with 25 ml. of iodine-free distilled water. After the last washing and centrifuging, the protein is dissolved in 5 ml. of 70 per cent H_2SO_4 and transferred to the digestion flask. Four further 5 ml. portions of acid and one final 5 ml. portion of water are employed to insure complete transfer.

2. Digestion—3 ml. of 60 per cent CrO_3 are added, plus a few glass beads, carborundum particles, or other antibumping agent. The digestion is carried out over a small flame until sulfuric acid fumes appear. The flask is allowed to cool, 15 ml. of water are added, and the digestion repeated.

3. Distillation—25 ml. of water and a few fresh beads are added and the chromic acid crystals dissolved by rotation just before distilling in order to make use of the heat generated by dilution. The flask is attached to the still and a micro burner flame placed beneath. Enough water is added through the upper opening of the trap to fill the region of attachment of the stop-cock, and then 0.5 ml. of a 10 per cent Na_2SO_3 solution is allowed to drain down the walls of the bulge above the trap. Most of this will collect in the low portion of the trap, and soon after the distillation of water vapor has begun, condensation results in the trap being completely closed with fluid.

The water-cooled condenser is connected and 5 ml. of 50 per cent H_3PO_3 are placed in the dropping funnel after it has been inserted into the free opening in the flask. All ground glass joints are lubricated with water before being assembled. After boiling has continued until water vapor has entered the condenser and has started to drip into the return tube, the H_3PO_3 is slowly blown into the flask by gentle pressure. The distillation is continued for 10 minutes after reduction is completed. To terminate the distillation, the flame is turned off and the trap is immediately drained into a 22 × 175 mm. test-tube calibrated at 25 ml. The condenser is raised clear and the walls of the trap rinsed down with five successive 2.5 ml. quantities of water, each washing being added to the distillate in the test-tube.

1 ml. of N H_2SO_4 is added to each combined distillate and washings. The resulting sulfurous acid is decomposed and SO_2 blown off by aeration at about 2.5 liters per minute while the tube is in boiling water. The tube is then cooled, the volume made to the 25 ml. mark, and the solution thoroughly mixed.

4. Colorimetric Determination of Iodide—5 ml. aliquots are pipetted from the 25 ml. total volume into Klett-Summerson colorimeter tubes. 0.4 ml. of arsenious acid is added, and the tubes are placed in a water bath accurately regulated to 37°. Two tubes containing 5.0 ml. of water and 0.4 ml. of arsenious acid should be routinely used for blank determinations in each series of twenty. Ceric ammonium sulfate is next added, but this must be done on a definite time schedule, since only one measurement is to be made of a rate of reaction. Incubation for the ceric sulfate-arsenious acid reaction is carried out for 15 minutes, and $\frac{3}{4}$ of a minute is allowed for each tube to be read in the colorimeter. At zero time, 0.5 ml. of the ceric ammonium sulfate solution is added to the first tube, the contents are quickly mixed, and the tube replaced in the water bath. A 45 second interval is allowed, and then the procedure is repeated for the rest of the tubes. Thus, a maximum of twenty individual tubes or ten duplicate determinations can be handled in one series. 15 minutes after addition of the ceric solution to the first tube, it is removed from the bath, the outside quickly wiped clean and dry, and a reading obtained in the photoelectric colorimeter with No. 42 blue filter. The same 45 second interval should be ample for making each reading.

Because of the necessity for rigid adherence to a time schedule, performance must be checked at the start by including standards in each series of determinations. Once the routine has been thoroughly established, it is adequate to run only one set of standards, ranging from 0.01 to 0.10 γ, as part of one series each time several sets of determinations are being performed.

The standard curve can be considered straight over only a restricted portion, and iodine values are best judged from an actual plot of reference values (Fig. 1). Blank values should be established for each new batch of reagents, and should be repeated occasionally as a check on contamination. After the blank has been deducted, calculations from these values should include the factor of 5 to cover the aliquot of 5 ml. out of the 25 ml. total volume and a factor of 50 to express the plasma PI in terms of micrograms per 100 ml. of plasma.

Comments and Precautions

1. Protein Precipitation and Washing—Somogyi's zinc precipitation procedure as described has been found by far the most convenient. Heat coagulation in a weakly acid medium is satisfactory, but requires constant attention. Trichloroacetic acid has been found unsatisfactory, at least partly because of the large amount of additional organic matter requiring digestion.

The precipitation technique has been applied successfully to tissues other than plasma by using the Potter homogenizer (3). 500 mg. of liver, kidney, heart, or skeletal muscle are homogenized in 8 ml. of the acid zinc sulfate reagent. This is poured into a 50 ml. centrifuge tube, followed by four rinsings of the grinder, each with 4 ml. of the reagent. 0.75 N NaOH is carefully stirred in, to a permanent pink with phenol red (about 3 ml. are required). When a normal thyroid gland is to be analyzed, it is homogenized in water or dilute H_2SO_4, the total volume of suspension plus rinsings being 100 ml. 1 ml. of the thoroughly mixed suspension is taken as an aliquot and is added to 2 ml. of dog plasma stock in a 50 ml. centrifuge tube. The Somogyi zinc precipitation is carried out as usual, followed by the four washings. In this case, the PI value of the dog plasma must be determined and deducted, together with the reagent blank value.

The four washings described have been found adequate to eliminate 99.92 per cent of 1000 γ of inorganic iodide added per 100 ml. of plasma and thus should offer ample routine protection. With the same procedure 5 γ per cent of added thyroxine iodine are retained completely by the precipitate, and 5 γ per cent of added diiodotyrosine iodine are retained to 75 per cent of completion. This curious situation has previously been noted by Man and coworkers (2).

2. Digestion—The amount of chromic acid has been increased over that recommended by Taurog and Chaikoff in order to insure an adequate excess for lipemic plasmas as well as for proteins of other tissues. Potassium or sodium dichromate can be used as well as chromic acid, but they are so much less soluble that the amount to be added would need to be used in solid form. Care should be taken that the second digestion is not prolonged beyond the stage of definite appearance of fumes, since excessive heating often results in considerable loss of the iodine present.

3. Distillation—In this laboratory the digestion of organic material has usually been carried out in ordinary 250 or 300 ml. Pyrex Florence flasks so that many determinations could be carried out without the expense entailed in an equal number of the special flasks used on the distillation apparatus. The principal inconvenience resulting from this economy is the insertion of an extra transfer from digestion flask to distillation flask by means of the 25 ml. of water, used in 5 ml. portions.

10 per cent sodium sulfite is used as the absorbing solution in the trap as a simple substitute for the Na_2CO_3-$NaHSO_3$ combination utilized by Talbot *et al.* Results in this laboratory with NaOH alone, as proposed by

Taurog and Chaikoff, have shown uniformly unsatisfactory recoveries, of the order of 0 to 10 per cent. The need for a reducing solution suggests that the volatile iodide may actually be iodine, instead of HI.

The duration of the distillation period is a compromise between the desire for a 100 per cent recovery and for as short a reaction time as possible. In one series of determinations, the recoveries of 0.10 γ of inorganic iodide added to digests of dog plasma were found to be as follows for various times: 5 minutes, 56 per cent; 7½ minutes, 78 per cent; 10 minutes, 85 per cent; 12.5 minutes, 89 per cent; 15 minutes, 92 per cent. At that rate, about 25 to 30 minutes would be required for a 98 to 100 per cent complete distillation. Since other series with 0.05 to 0.25 γ of I have shown 87 to 95 per cent recoveries in 10 minutes, this time has been selected. A correction factor covering the 5 to 15 per cent loss may be introduced if desired.

Although the sodium sulfite has been found essential, as noted earlier, it must be decomposed to SO_2 by acid and this blown off in order not to cause a complete and non-specific decolorization of the ceric solution later. Compressed air has been used, since the substitution of N_2 did not alter the results. A simple and effective set-up consists of a four or six outlet manifold, with finely drawn out glass tubing which reaches nearly to the bottom of each tube. The tubes specified are long enough so that vigorous bubbling can be accommodated. A screw clamp control should be provided for each nozzle to prevent excessive air currents. This aeration step also results in some volatilization of water; so that the level in each tube should be less than 25 ml. even after adequate rinsing of the fine glass tubes. Thus, ample allowance is made for the addition of water to a consistent final volume.

4. Colorimetric Determination of Iodide—This part of the determination is the one most apt to present difficulties, owing to the need for rigorous control of nearly every phase. However, with the requisite amount of care, highly dependable results can be obtained, as is shown by Fig. 1.

Time must be controlled to a far greater extent than is usual with colorimetric procedures, inasmuch as differing rates of a reaction eventually proceeding to completion are involved. Those who have previously used the ceric sulfate-arsenious acid reaction have taken two, and often more, readings in order to establish definitely the rate of decolorization. However, in the interests of simplification, it has seemed desirable to acknowledge the arbitrariness of all colorimetric procedures and to make only a single routine reading at 15 minutes. This should be supplemented by a 30 minute reading whenever the first reading shows a decolorization amounting to less than 40 units on the Klett-Summerson colorimeter (yielded by 0.005 γ of I) if one desires the most accurate results possible in the lower range of iodide values.

Since accurate temperature control is also necessary during the incubation, it has been found convenient to employ a test-tube rack of appropriate size suspended from hooks hung on the opposite sides of a constant temperature water bath maintained at 37°. Although this was done to avoid the nuisance of continually resetting the thermoregulator, any temperature in the region of 30–40° is satisfactory, provided it is regulated within ±0.1°.

The ceric ammonium sulfate solution is measured with the greatest possible accuracy, since it contributes the initial amount of color. The 0.5 ml. required is delivered from a fine tipped, small orifice pipette, between two marks. There is little need for accurate standardization of the solution from an oxidation-reduction standpoint, because each new stock will require that a blank be established and that the iodine effect be standardized.

TABLE I

Effect of Sodium Chloride on Iodide Catalysis of Ceric Sulfate Decolorization

Iodine	NaCl, mg.						
	0.0	1.0		2.0		3.0	
	Colorimeter reading	Colorimeter reading	Increase*	Colorimeter reading	Increase*	Colorimeter reading	Increase*
γ			*per cent*		*per cent*		*per cent*
0	490	488		486		485	
0.010	445	422	55	410	82	411	82
0.025	359	319	35	304	47	303	47
0.050	241	199	23	184	34	174	35
0.100	86	50	27	43	31	43	31

* This was calculated on the basis of the iodine values of the colorimeter readings without NaCl.

The 45 second interval between tubes has proved the most valuable, 30 seconds being too short an allowance for all colorimeter readings to be taken and 60 seconds unnecessarily long. One control blank in duplicate plus nine determinations in duplicate on distillates (twenty tubes in all) can thus be handled successfully in each series. The photoelectric colorimeter to be used should be adequately tested for adjustment immediately before one starts the addition of ceric sulfate.

Table I shows the considerable enhancement of the iodide catalysis caused by the inclusion of 1 to 3 mg. of sodium chloride per tube. The maximum effect appears to be exerted on the smaller amounts of iodide, being as great as 82 per cent with 0.010 γ of iodine. Quite satisfactory, reproducible results can thus be obtained with 0.005 γ of I. That contamination of the salt with iodide is not giving a spurious "catalysis" is evident from inspection of the data in Table I: the blank values on the reagents show only a slight extra decolorization with increased amounts of chloride; the results with 2.0 and with 3.0 mg. of added NaCl are essentially the same.

Results

Although the standardization of the iodide-catalyzed arsenious acid decolorization of the yellow ceric solution requires the extraordinary precautions already discussed, remarkably consistent results are obtained when these precautions are scrupulously observed. Fig. 1 shows the mean and the maximum deviation from the mean of points on a calibration curve; ten duplicate determinations were performed on each point over a period of 3 months. From the 30 minute curve in Fig. 1 it can be seen that reliable results have even been obtained with 0.005 γ of iodide under these circumstances.

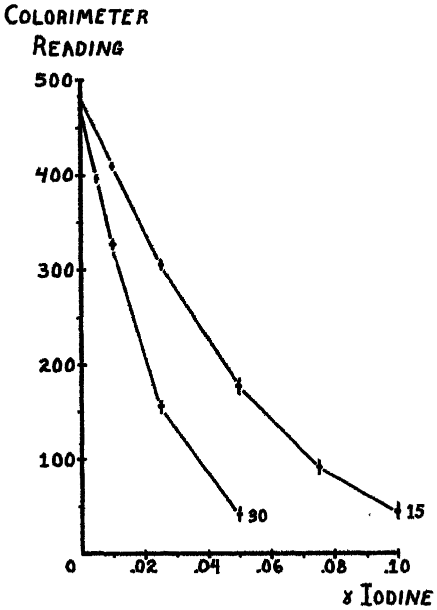

FIG. 1. Relationship between the Klett-Summerson colorimeter reading and standard amounts of iodine. The incubation time is indicated on each curve as 15 or 30 minutes.

In order to test the application of this sensitive analytical procedure to biological material, aliquots of dialyzed, pooled dog plasma were analyzed as such and after the addition of amounts of iodide, thyroxine, or diiodotyrosine ranging from 0.05 to 0.50 γ (as I). Table II shows that recoveries were 87 to 95 per cent complete when the addition was made before distillation was carried out, whereas they were 98 to 101 per cent when iodide was added to the distillate. These findings strongly indicate some loss of iodide during distillation, probably due to the arbitrary termination of this step at 10 minutes. As previously mentioned, it was not thought worth while to continue the distillation to the point of a more complete recovery. In this laboratory, a 10 per cent correction is routinely made.

TABLE II

Recovery of Iodine from Compounds Added to Dog Plasma

Substance added	Added amount	Amount recovered*	
	γ	γ	*per cent*
NaI, before digestion	0.10	0.088	88.0
“ “ “	0.25	0.226	90.4
Thyroxine, before digestion	0.10	0.093	93.0
“ “ “	0.20	0.181	90.5
Diiodotyrosine, before digestion	0.10	0.090	90.0
NaI, after digestion	0.05	0.045	90.0
“ “ “	0.10	0.092	92.0
“ “ “	0.25	0.217	86.8
“ “ “	0.50	0.439	87.8
KIO_3, “ “	0.10	0.085	85.0
“ “ “	0.25	0.236	94.4
NaI, “ distillation	0.10	0.101	101.0
“ “ “	0.25	0.244	97.6

* All figures are the averages of at least four duplicate determinations.

TABLE III

Protein-Bound Iodine in Tissues of Various Species

Species	Tissue	No. of animals	Protein-bound iodine
			γ per 100 ml.
Rat, normal	Blood plasma	28	4.2 ± 0.4*
“ on thiouracil	“ “	23	1.1 ± 0.2
“ “ thyroxine	“ “	15	18.0 ± 1.0
Dog, normal	“ “	8	2.3
Human, normal	“ “	6	7.5
Rat, normal	Thyroid	7	23.3†
“ “	Liver	2	30.6
“ “	Kidney	2	35.5
“ “	Heart	2	26.6
“ “	Skeletal muscle	2	26.7

* Standard deviation.

† Micrograms of PI in the entire thyroid gland (10 to 20 mg.).

Another fact which should be pointed out is the poor recovery of inorganic iodide in the absence of organic material to be digested. Recoveries under such circumstances range from 70 to 85 per cent. Taurog and Chaikoff also noted this, and reported the use of iodine-free wheat as an organic carrier. In this laboratory we have found dialyzed dog serum much more convenient; it can be stored in the frozen condition for long periods of time or it can be lyophilized and redissolved when needed. The iodine values are low and remain stable over long periods of storage in the frozen or dried state.

Studies on the various solutions obtained at different stages of the procedure have revealed unexpected keeping qualities, provided bacterial contamination does not occur. Plasmas and final distillates have been kept in the refrigerator without preservative for as long as 3 months without a detectable loss of iodide. Simple water solutions of NaI containing as little as 0.01 γ of I per ml. have remained stable for 10 months.

Table III contains the results of estimations of protein-bound iodine in blood plasma of three species, as well as in various tissues of the rat. In all instances, inorganic iodide was washed out. When thyroxine or diiodotyrosine was added in amounts equivalent to 1.0 to 5.0 γ per 100 ml. of plasma before the washing, recoveries of approximately 100 and 75 per cent (corrected) respectively were obtained. These findings indicate adsorption of thyroxine and, to a somewhat lesser extent, diiodotyrosine on the zinc proteinate precipitate. The binding must be strong to withstand four washings.

SUMMARY

A procedure has been reported for the determination of protein-bound iodine in various tissues, including blood plasma. The principal steps are precipitation, washing and then oxidation of the protein, distillation of the iodine, and colorimetric determination of the iodine by means of its catalytic effect on the reduction of ceric ions by arsenious acid.

The present method, combining and modifying previous methods, permits a satisfactory analysis to be performed on 2 ml. of plasma, one-hundredth of a normal rat thyroid gland, or 500 mg. of rat liver, kidney, heart, or skeletal muscle.

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THE SYNTHESIS OF FATTY ACIDS IN ADIPOSE TISSUE IN VITRO

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The conversion of carbohydrate into fatty acids in the animal body has been clearly demonstrated by Schoenheimer and Rittenberg (1) and by Bernhard and Schoenheimer (2) with the aid of deuterium oxide. The D_2O concentration of the body fluids was raised and the deuterium introduced into the fatty acids was taken as a measure of the newly synthesized fatty acids.

It is generally assumed that the liver is the sole site of this synthesis. However, a quantitative analysis made by Stetten and Grail (3) is not easily reconciled with this assumption. According to their results, it has been calculated that the quantity of fatty acids synthesized per day by a mouse is about 4 times the quantity present in the liver. If the liver is assumed to be the major site of synthesis, the half life time of fatty acids in the liver should be several hours, whereas the half life time actually found was between 2.6 and 2.8 days. Tepperman, Brobeck, and Long (4) produced evidence that carbohydrate can be converted into fat in the extrahepatic tissues. They were able to show that fatty acid synthesis was augmented in rats on a dietary training, and that this augmentation persisted in the functional absence of the liver.

Tuerkischer and Wertheimer (5) suggested that adipose tissue may play a part in the synthesis of fat. They showed that if, after rats have been fasted or given inadequate diets long enough to exhaust their stores of fat, the animals are realimented with high carbohydrate diets, glycogen is initially deposited in the fat cells. Only after an interval has elapsed does the glycogen gradually disappear, giving way to fat. In earlier experiments (6), it had been found that in all conditions favoring fat synthesis from carbohydrates, fat deposition in adipose tissue is preceded by the appearance of glycogen in this tissue. It was furthermore shown by Mirski (7) that isolated, glycogen-containing adipose tissue has a respiratory quotient of 1.1 to 1.3, whereas ordinary, glycogen-free adipose tissue has a respiratory quotient of 0.70. The respiratory quotient of glycogen-laden adipose tissue was especially high and most consistent when the tissue was incubated in serum. It is thus presumable that adipose tissue is capable of synthesizing fatty acids.

In order to test this assumption, the method of Schoenheimer and Rittenberg (1) was used with adipose tissue incubated *in vitro*. Deuterium oxide was added to the medium and the deuterium found in the isolated fatty acids was taken as a measure of synthesis of fatty acids. Experiments were carried out with mesenteric fat, interscapular fat, and groin fat of rats on dietary régimes inducing fat synthesis and deposition of glycogen in adipose tissue, on the one hand, and of rats on a stock diet, on the other hand.

Table I

Introduction of Deuterium into Fatty Acids of Adipose Tissue

Tissue	Treatment of rats	Fatty acids per 100 gm. body weight	Deuterium concentration	
			Atom per cent excess in fatty acids	Per cent ratio in fat to that in medium
		mg.		
Mesenteric fat	Stock diet	145	0.020	0.22
" "	" "	180	0	0
" "	Diet 1	130	0.022	0.23
" "	" 1	122	0.025	0.27
" "	" 2	95	0.046	0.51
" "	" 2	105	0.040	0.44
Interscapular fat	Stock diet	85	0.034	0.38
" "	" "	80	0.052	0.57
" "	Diet 1	70	0.064	0.71
" "	" 1	75	0.056	0.62
" "	" 2	70	0.021	0.23
" "	" 2	68	0.074	0.81
Groin fat	Stock diet		0.012	0.13
" "	Diet 1		0.018	0.20
" "	" 2		0.023	0.25
" "	" 2		0.019	0.21
Liver slices*	Diet 1	88	0.062	1.75
" "	" 2	82	0.070	2.00

* Liver slices were incubated in serum with only 5 per cent deuterium oxide, making a final deuterium concentration in the tissue fluid of about 3.5 per cent.

EXPERIMENTAL

Adipose tissue was taken from rats on a stock diet and from rats maintained on the following dietary régimes: In Diet 1 food intake was restricted for 5 to 7 days until a weight loss of 20 per cent resulted, followed by subsequent feeding *ad libitum* for 48 hours on a diet consisting of 70 per cent carbohydrates, 20 per cent casein, and 10 per cent oil. Diet 2 differed from Diet 1 only in that rats were kept on the restricted diet until 30 per cent weight loss resulted and were then refed for 16 hours only. Adipose tissue of the rats on Diets 1 and 2 contained glycogen in a concentration of 0.2 to 0.5 per cent.

Tissues were removed, finely cut with scissors, and incubated for 4 hours at 37° in 3 volumes of rat serum enriched with deuterium oxide to a concentration of about 10 atom per cent. After incubation, the fat was saponified with 20 per cent KOH, acidified, and the fatty acids extracted with ether. After evaporation of the ether, the residue was dissolved in aqueous 5 per cent KOH (in order to eliminate the carboxyl deuterium), acidified, and re-extracted with ether. The ether was dried with sodium sulfate and evaporated. The deuterium concentration in the fatty acids thus isolated was estimated by the method of Keston, Rittenberg, and Schoenheimer (8).

Results

The results, summarized in Table I, show that deuterium is introduced into the fatty acids of adipose tissue incubated in serum enriched with deuterium oxide. The deuterium concentration is generally higher with adipose tissue from rats in a condition of accelerated fat synthesis (Diets 1 and 2) than with adipose tissue from normal rats. With normal groin fat, the deuterium concentration found in the fatty acids was very low, owing to the high initial fat content. Higher values are found when part of the fat has previously been depleted (Diet 2).

The experiments were carried out in serum as a medium, since in this medium the most consistently high respiratory quotients were found (7). Several experiments carried out in Ringer's solution gave similar but less marked results.

DISCUSSION

The results confirm the assumption that adipose tissue is capable of synthesizing fatty acids. The higher activity found in glycogen-containing adipose tissue (Diets 1 and 2) suggests that glycogen may play a rôle in the synthesis of fatty acids. The results do not as yet permit an exact evaluation of the part played by adipose tissue in fat synthesis in the animal body. When adipose tissue and liver are compared, it is found that liver is more active in renewing its fatty acids by synthesis. Since, however, the total amount of fatty acids in adipose tissue is many times greater than that in liver, adipose tissue may be responsible for a high percentage of the total quantity of fat synthesized in the body, in spite of the slower overturn in this tissue.

SUMMARY

Adipose tissue incubated *in vitro* in serum enriched with deuterium oxide introduces deuterium into its fatty acids. The rate of introduction is greater in adipose tissue of rats on a diet accelerating fat synthesis in the body.

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GLUTAMINE, AN ANTIMETABOLITE FOR STAPHYLOCOCCUS AUREUS

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In a previous communication (1) it was shown that the antibacterial effect of L- and DL-N-(γ-glutamyl)-ethanolamine and DL-N-(γ-glutamyl)-ethylamine upon *Staphylococcus aureus* could be reversed by glutamic acid. Preliminary data (1) showed that glutamine was unable to prevent this inhibition. The ineffectiveness of glutamine in overcoming the inhibition seemed to be rather curious, since glutamine is known to be a growth factor for at least two organisms, *Streptococcus pyogenes* (2, 3) and *Neisseria gonorrhoeae* (4), and since it has furthermore recently been shown to counteract the inhibition of growth of *Lactobacillus arabinosus* caused by a sulfoxide derived from methionine (5, 6).

Further experiments were therefore undertaken to reinvestigate this problem. Data will be presented to show that glutamine, alone, very markedly inhibits the growth of our previous test organism, *Staphylococcus aureus*, and that this inhibition is specifically reversed by L-glutamic acid.

EXPERIMENTAL

Methods—We followed the technique previously described (1) with respect to the composition of the medium, the test organism, incubation periods, and growth estimation. We used as an inoculum 0.1 ml. per tube of a 100-fold dilution of a barely turbid suspension of staphylococci. Of the test materials used, glutamine and glutathione were sterilized by filtration and others by autoclaving. The sterile ingredients were added aseptically to the medium, and the volume was made up to 5 ml. with sterile water. Growth was measured turbidimetrically with an Evelyn type electrophotometer. Since the experiments were carried out in specially matched tubes, the estimation of turbidity could be made at any stage of growth, and our final measurement was made after 48 hours; more prolonged incubation did not alter the results.

Effect of Glutamine on Staphylococcal Growth—Table I shows that glutamine inhibits the growth of *Staphylococcus aureus* to a very marked degree. As little as 3 mg. of glutamine per 5 ml. of medium inhibits growth by 80 to 90 per cent, and an inhibition of about 50 per cent is caused by 2 mg. Lower concentrations produced less marked inhibition, whereas higher ones did not produce complete bacteriostasis. A similar effect was found with the glutamic acid alkylamines (1). However, glutamine is 4 to 5 times more potent than the two alkylamines, L- and DL-N-(γ-glutamyl)-ethanolamine and DL-N-(γ-glutamyl)-ethylamine, previously tested.

Since the glutamine preparation used is not a synthetic product and may therefore be contaminated with biologically active substances such as arginine (7), it seemed necessary to determine whether the inhibitory effect was really due to glutamine itself or to a possible associated by-product. Experiments were carried out to rule out the latter possibility. (1) Different batches of glutamine, namely two batches of glutamine from General Biochemicals, Inc., as well as a batch of glutamine from The British Drug Houses, Ltd., gave identical results. (2) Arginine had no effect whatsoever. (3) The inhibition is of a competitive character and is specifically counteracted by L-glutamic acid. An almost constant ratio was found to exist between the inhibitor (glutamine) and the metabolite (glutamic acid) (Table II).

TABLE I

Effect of Glutamine on Growth of Staphylococcus aureus

Compound added	Concentration	Photometer reading after 48 hrs. growth*
	mg. per 5 ml.	
None		45
"		46
Glutamine	0.5	46
"	1.0	52
"	2.0	64
"	3.0	78
"	4.0	88
"	5.0	90
"	10.0	87
None (uninoculated)		100

* A reading of 100 indicates no growth. Increased growth is reflected by a decreased reading.

We are thus obviously dealing with a specific inhibition due to glutamine.

Specificity of L-Glutamic Acid in Reversing Glutamine Inhibition—We next attempted to elucidate the mechanism by which glutamine inhibits the growth of *Staphylococcus aureus*. A series of compounds structurally related to L-glutamic acid was chosen: L-asparagine and the sodium salts of DL-aspartic acid, DL-α-aminoadipic acid, and L-pyrrolidonecarboxylic acid. The results are summarized in Table III. It may be seen that, although some of these substances stimulated the growth of the organism to a slight degree, none of them, except L-glutamic acid, was capable of abolishing the inhibition caused by glutamine.

The specificity of L-glutamic acid as an antagonist of glutamine is even more pronounced than would appear from the above. When the natural optical isomer is replaced by the racemic DL-glutamate, the latter, instead of showing the expected 50 per cent activity, as compared with the L antipode, was found to be completely inactive (Table III). This experiment was repeated several times with various preparations of DL-glutamic acid (Merck and Company, Inc., Hema Drug Company, Inc., and a sample prepared by Dr. N. Lichtenstein, The Hebrew University, Jerusalem). The same results were regularly obtained even when concentrations 5 times as great as the effective concentration of L-glutamic acid were used. DL-Glutamic acid does not actually inhibit growth, but is merely ineffective in restoring growth in the presence of the inhibitor (glutamine).

TABLE II

Inhibition of Growth of Staphylococcus aureus by Glutamine, and Its Reversal by L-Glutamic acid

Glutamine concentration	L-Glutamic acid concentration	Photometer reading after 48 hrs. growth	$\frac{\text{Glutamine}}{\text{L-Glutamic acid}}$ at 50 per cent inhibition
mg. per 5 ml.	*mg. per 5 ml.*		
0	0	47	
0	4	45	
4	0	86	
4	0.2	86	
4	0.5	72	8
4	1.0	50	
8	0	86	
8	0.5	85	
8	1	77	6 *ca.*
8	2	67	
8	3	48	
12	0	87	
12	1	82	
12	2	71	6 *ca.*
12	3	68	
12	4	56	

Effect of Pteroylglutamic Acid and Glutathione on Inhibition of Growth by Glutamine—Since none of the compounds related to L-glutamic acid was able to reverse the inhibition caused by glutamine, we tried the effect of two natural tripeptides containing L-glutamic acid as a component, (1) pteroylglutamic acid (folic acid), in which the glutamic acid is bound through its amino group, and (2) glutathione, in which the glutamic acid is bound through its γ-carboxyl, as in glutamine and in the γ-glutamylamines. Table IV shows that both tripeptides are highly efficient in reversing the inhibitory effect of glutamine. On the basis of glutamic acid content, folic acid (33 per cent glutamic acid) is about 15 to 20 times as active as L-glutamic acid and glutathione (48 per cent glutamic acid) is about 10 to 12 times as effective as L-glutamic acid.

TABLE III

Effect of Various Compounds Related to Glutamic Acid on Inhibition of Growth of Staphylococcus aureus by L-Glutamine

Inhibitor	Glutamic acid* and related compounds	Concentration mg. per 5 ml.	Photometer reading after 48 hrs. growth
None	None		43
"	L-Glutamic acid	5	43
"	DL-Glutamic "	5	43
"	" "	15	46
"	DL-Aspartic "	5	42
"	" "	15	47
"	L-Asparagine	5	37
"	"	15	41
"	DL-α-Aminoadipic acid†	5	42
"	" "	15	41
Glutamine, 5 mg. per 5 ml.	None		87
" "	L-Glutamic acid	0.5	65
" "	" "	2	42
" "	" "	5	38
" "	DL-Glutamic "	5	85
" "	" "	10	82
" "	" "	15	78
" "	DL-Aspartic "	5	82
" "	" "	15	85
" "	L-Asparagine	5	82
" "	"	15	85
" "	DL-α-Aminoadipic acid	5	81
" "	" "	15	83
" "	L-Pyrrolidonecarboxylic acid	10	84
" "	" " Ammonium chloride	10 10	79

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* All the acids were assayed as neutral sodium salts.

† We are indebted to Dr. Heinrich Waelsch, Columbia University, New York, for the sample of DL-α-aminoadipic acid.

DISCUSSION

Glutamic acid plays an important rôle in the metabolism of *Staphylococcus aureus.* This organism grows luxuriantly in a synthetic amino acid medium devoid of glutamic acid, apparently synthesizing the required amino acid (8). If glutamic acid is added to the medium, the staphylococci assimilate it and concentrate it in their protoplasm (9). Our strain behaved normally in this respect in that it was capable of building this amino acid by itself. Now, if sufficient glutamine is added to a casein hydrolysate medium which contains glutamic acid, the growth is inhibited, and extra glutamic acid is required to overcome the inhibitory effect. This inhibition is very specific. Neither the 4-carbon analogues, DL-aspartic acid and L-asparagine, nor the 6-carbon analogue, DL-α-aminoa-

TABLE IV

Effect of L-Glutamic Acid, Pteroylglutamic Acid, and Glutathione on Inhibition of Growth of Staphylococcus aureus by Glutamine

Inhibitor	Glutamic acid and derivatives	Concentration	Photometer reading	
			24 hrs.	48 hrs.
		mg. per 5 ml.		
None	None		51	40
“	“		54	42
“	L-Glutamic acid	2	66	42
“	Pteroylglutamic acid*	0.5	63	39
“	Glutathione	1	61	43
Glutamine, 4 mg. per 5 ml.	None		85	84
“ “	L-Glutamic acid	0.1	82	83
“ “	“ “	0.3	84	84
“ “	“ “	0.6	75	63
“ “	“ “	1	62	48
“ “	“ “	2	55	39
“ “	Pteroylglutamic acid	0.03	80	78
“ “	“ “	0.1	75	57
“ “	“ “	0.3	73	42
“ “	“ “	0.5	72	41
“ “	Glutathione	0.03	87	84
“ “	“	0.1	82	60
“ “	“	0.3	76	51
“ “	“	0.6	76	47
“ “	“	1.0	71	42

* Synthetic pteroylglutamic acid (folic acid) has been obtained through the courtesy of Dr. S. M. Hardy, Lederle Laboratories Division, American Cyanamid Company.

dipic acid, has any effect upon it. L-Pyrrolidonecarboxylic acid and its ammonium salt are also without effect. Furthermore, the racemic DL-glutamic acid, though not in itself inhibitory, is incapable of counteracting the inhibition due to glutamine.

The ineffectiveness of DL-glutamic acid is striking. Instead of showing the expected 50 per cent activity, it is not effective at all. The inactivity of racemic glutamic acid may be explained in either of two ways: The D antipode may show inhibiting properties comparable in extent to the counteracting effect of the L antipode on glutamine, and these two effects may cancel each other. It is possible, also, to assume the formation of a stable racemate, the antipodes of which have greater affinity for each other than that of the enzyme for the L form (10). This problem is now under investigation.

L-Glutamic acid and the two natural tripeptides, glutathione and folic acid, are the only ones in a series of substances tested which reverse the inhibition of staphylococcal growth by glutamine. In folic acid, the glutamic acid is bound through its amino group, whereas in glutathione it is bound through the γ-carboxyl as in glutamine and in the γ-glutamylamines. It is, therefore, rather surprising that glutathione is so efficient in overcoming the inhibition by glutamine. It is interesting to note that Waelsch, Owades, Miller, and Borek (6) also observed that glutathione behaves like glutamic acid and not at all like glutamine towards the sulfoxide of methionine.

These results may best be interpreted by assuming that we are dealing with an inhibition of the competitive type. Glutamine, closely related to glutamic acid, possesses great affinity for that enzyme system which normally combines with glutamic acid. Since the staphylococci are apparently devoid of glutaminase, the glutamine is not broken down, and reactions essential for glutamic acid metabolism and growth are thereby blocked. The resulting inhibition is, however, completely reversible, since growth is resumed on the addition of glutamic acid even if bacteriostasis has already taken place.

The fact that glutamine is an efficient antimetabolite seems remarkable to us. The great majority of the numerous structural analogues which show antagonistic properties towards their mother substances are artificially changed compounds. Some substances, however, are naturally occurring metabolites (*e.g.* asparagine, which is antagonistic to β-alanine in a yeast (11), isoleucine antagonistic to leucine in *Pasteurella pestis* (12), and arginine antagonistic to lysine in *Neurospora* (13)).

Furthermore, our data illustrate the fact that under certain conditions an essential metabolite and growth factor may show inhibitory properties. The inhibiting concentration of glutamine is, however, higher than that required for growth stimulation in sensitive organisms, ranging as it does from 0.06 mg. per 100 ml. in *Lactobacillus casei* (14) to 0.03 mg. and even to 20 mg. per cent in *Streptococcus hemolyticus* (2, 3), whereas an inhibition of about 50 per cent is obtained only by 40 mg. per cent. Nevertheless, in spite of the high concentrations of the glutamine this is a specific inhibition governed by the laws of competitive interference with the essential metabolite.

The question of the identity of the metabolite (whether it is the glutamic acid itself or some glutamic acid peptide) also merits mention. Since both glutathione and folic acid are considerably more effective than glutamic acid in overcoming the growth inhibition produced by glutamine, one might infer that these two substances are actually the end-products of glutamic acid metabolism, the formation of which is inhibited by glutamine. This inference is to a certain extent, though not conclusively, contradicted by the data in Table IV. Each of these three substances, L-glutamic acid, folic acid, and glutathione, when added to the assay medium, causes a prolonged lag phase, a transient inhibition which disappears on further incubation (compare 24 and 48 hour data in Table IV). The possibility is therefore not excluded that glutamic acid is converted not into one of these peptides but into another substance of similar structure.

SUMMARY

The inhibiting effect of glutamine upon *Staphylococcus aureus* is demonstrated. This inhibition is of a competitive type and is specifically reversed by L-glutamic acid, pteroylglutamic acid, and glutathione. Substances like DL-aspartic acid, L-asparagine, DL-α-aminoadipic acid, and L-pyrrolidonecarboxylic acid are ineffective.

The unexpected ineffectiveness of DL-glutamic acid in overcoming the inhibition is discussed.

The inhibition indicates the absence of a glutaminase capable of converting glutamine into glutamic acid.

The rôle of pteroylglutamic acid and glutathione in the metabolism of *Staphylococcus aureus* is briefly discussed.

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THE DISTRIBUTION AND EXCRETION OF INJECTED URANIUM*

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The distribution and excretion of injected uranium have been the subject of a number of papers (1–9) wherein a variety of analytical methods was employed and a variety of conclusions reached. For example, some authors (6, 8) considered the liver as an important site of uranium deposition, while others (3, 4) reported the liver to be uranium-free. Some (1, 3) found uranium to be excreted in the bile, a fact denied by others (7).

All of these investigators agreed that uranium accumulates in the kidneys and most of the recent publications indicated that large quantities of injected uranium were excreted in the urine. None of these investigators[1] examined bone as a possible site of storage, in spite of the fact that Hoffmann (10) found traces of uranium in the bones of normal, untreated animals.

In the experiments reported here, balance studies were performed on rats; in addition, corollary studies with other species were conducted to clarify certain points. Injected uranium was found to be rapidly excreted in the urine, very little appearing in the feces. Bone and kidney were the only tissues which retained significant quantities.

EXPERIMENTAL

Methods

Because of the influence diet might have on the distribution and excretion of uranium, a synthetic ration, which could be defined and reproduced, was chosen. Rats kept on this ration for several months grew well and evidenced no deficiency symptoms.

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* This paper is based on work performed under contract No. W-7401-Eng-49 for the Manhattan Project at the University of Rochester.

[1] Exceptions can be found in the classified literature of the Manhattan Project. A comprehensive study of the distribution and excretion of injected and ingested uranium was reported by Tannebaum, A., Silverstone, H., and Koziol, J., Chicago Atomic Energy Report No. CH-3659; similar experiments with uranium given by intratracheal insufflation were reported by Thompson, H. E., and Steadman, L. T., Rochester Atomic Energy Report No. M-1984. The results of these independent studies are in excellent agreement with the data reported here.

This diet had the following composition, in parts by weight: 25 casein, 40 starch, 10 sugar, 5 yeast, 4 alfalfa, 20 Cellu flour, 7.5 lard, 2.5 cod liver oil, 4.9 salt mixture (11), 1.0 calcium carbonate, 0.1 Natola (fish oil concentration), 0.2 choline chloride, and 0.21 vitamin mixture.

The vitamin mixture had the following composition in mg.: thiamine 80, riboflavin 160, pyridoxine 120, nicotinic acid 250, calcium pantothenate 250, *p*-aminobenzoic acid 230, inositol 500, and copper sulfate 500.

The high Cellu flour content of the diet initially produced a mild diarrhea lasting 1 or 2 days. Because of this, all experimental rats were given a minimal equilibrating period of 1 week on the ration before uranium was administered.

Male and female Wistar rats weighing 200 ± 5 gm. were injected intravenously (by tail vein) with a solution of 0.1 per cent uranyl nitrate (hexahydrate) at a dose level of 2.5 mg. of U per kilo. The animals were placed in individual, all-glass metabolism cages which permitted separate urine and feces collection. Each day, the cages were washed thoroughly with 2 N HCl solution containing 0.1 per cent detergent (special sample of acid-stable detergent, Igepal CTA extra, kindly furnished by the General Dyestuff Corporation, New York). After specified intervals of from 45 minutes to 44 days after injection, the animals were anesthetized lightly with ether, blood being collected by aspiration from an axillary pouch made according to Kuhn (12), and were then sacrificed. Dissections were made with scrupulously clean instruments which were kept under a 2 per cent solution of sodium bicarbonate when not in use. The following samples were taken: kidney, urinary bladder, spleen, gonads, heart, lungs, liver, stomach and contents, intestines, leg muscle, skin and hair, femurs and humeri, tibiae, radii, fibulae, ulnae, pelvic girdle, three or four vertebrae, ribs, skull, and tail. The rest of the animal was divided into two samples, soft carcass and bone carcass; each bone was scraped free of adhering flesh. A complete balance record, *i.e.* uranium administered *versus* uranium recovered, was kept for each rat.

Corollary experiments were conducted on other species. In these studies, the animals were anesthetized and infused with fluids as specified, the bladders were cannulated, and, after a single intravenous injection of uranyl acetate, urine samples were collected over varying periods from 4 to 8 hours.

All analyses for uranium were made by the fluorophotometric method described previously (13).

Results

Uranium Content of Normal Tissue—Two normal, untreated rats were sacrificed, dissected, and analyzed as described above to establish the range of concentrations of uranium in normal tissue. The results are given in Table I.

The concentrations observed in normal rats were less than 0.1 γ per gm. of tissue, in agreement with the results of Hoffmann (10). Actually, it is doubtful whether these data represent the true tissue content; rather, they represent a measure of the extent to which contamination was controlled.

In the data to follow, only concentrations greater than 0.1 γ per gm. of tissue were considered significant.

TABLE I

Uranium Content of Normal Rat Tissues

Tissue	Rat 1			Rat 2		
	Tissue wt.	Total U*	U concentration	Tissue wt.	Total U*	U concentration
	gm.	γ	γ *per gm.*	*gm.*	γ	γ *per gm.*
Carcass	47.55			71.40	0.00	0.00
Muscle	42.00	0.38	0.01	27.21	0.00	0.00
Skin and hair	33.09	0.45	0.01	31.15	0.39	0.01
Intestines	17.66	0.12	0.01	20.32	0.13	0.01
Liver	8.72	0.22	0.04	9.65	0.00	0.00
Blood	7.03	0.14	0.02	7.77	0.08	0.01
Stomach	4.82	0.04	0.01	1.55	0.02	0.01
Skull	2.71	0.00	0.00	2.97	0.00	0.00
Genitals	2.38	0.00	0.00	2.54		
Lungs	1.15	0.03	0.03	1.03	0.00	0.00
Vertebrae	1.06	0.03	0.03	1.21	0.00	0.00
Tibiae	1.26	0.00	0.00	0.85	0.00	0.00
Kidneys	1.63	0.03	0.02	1.66	0.00	0.00
Femurs	0.79	0.00	0.00	1.01	0.00	0.00
Spleen	1.16	0.03	0.03	1.35	0.01	0.01
Heart	0.81	0.06	0.07	0.82	0.06	0.07
Pelvic girdle	0.67	0.00	0.00	0.75	0.00	0.00
Ribs	0.41	0.03	0.07	0.35	0.00	0.00

* Total uranium values less than 0.05 γ are of doubtful significance (13).

Analytical Recoveries—To prevent analytical inaccuracies from giving misleading results in the experiments below, a balance study was conducted on each rat examined. A summary of these results is presented in Table II. Included in the table are the results of control isolations from pure uranyl nitrate solutions carried out simultaneously with isolations from tissue samples.

It should be pointed out that, in these experiments, most errors were positive. For example, of a series of analyses of normal urine specimens which are uranium-free, some will give slightly positive results, some true blanks, and some slightly negative results. However, all negative results are reported "zero," giving an average which is positive. Therefore, the highest recoveries were observed in those animals from which the greatest number of samples were taken (greatest accumulation of positive errors). In practice, it was the long term animals from which the greatest number of specimens were taken (daily urine and feces collections over periods of 10 to 40 days). Accordingly, the results obtained from the long term animals have been listed separately in Table II.

TABLE II

Over-All Recoveries in Balance Experiments

	No. of samples	Mean recovery	Standard deviation
		per cent	*per cent*
Uranyl nitrate solutions	38	85	8
Short term animals (14)	400	90	7
Long " " (7)	420	108	13

TABLE III

Variation in Uranium Concentration in Rat Blood with Time

Time after injection	Blood uranium level	
	Females	Males
hrs.	*γ per ml.*	*γ per ml.*
0 (Calculated)*	36	36
0.75	1.5	1.3
2.5	0.2	0.0
8		0.1
12	0.1	0.1
24	0.0	0.1
48	0.1	0.0
960	0.0	0.1

* Calculated on the assumption that the blood volume constituted 7 per cent of the total body weight.

The data show that the recovery of uranium from the animal was, in final analysis, equivalent to that obtained with pure solutions of uranyl nitrate.

Blood—Analyses of blood have been made on two species, rats and rabbits; the literature contains one report on dogs (8). All three species evidenced a rapid disappearance of uranium from the blood after injection.

The data obtained with rats are summarized in Table III. Fig. 1 presents graphically the results of the experiments on rabbits, including one in which the rabbit had been nephrectomized.

In rats and normal rabbits, over 99 per cent of the administered uranium had left the blood stream in 2 hours. Even nephrectomy did not markedly delay the process. In dogs, as reported by Holman and Douglas (8), uranium left the circulation somewhat more slowly. However, the dogs received uranium intraperitoneally.

Urinary Excretion—Data obtained from three species, rats, cats, and rabbits, indicated that the urinary excretion of uranium is also rapid. The averaged urinary excretion of rats is presented in Fig. 2. Most of the excretion took place in the first 24 hours after administration. Practically all of the excreted uranium was found in the urine. Holman and Douglas (8) obtained similar results with dogs.

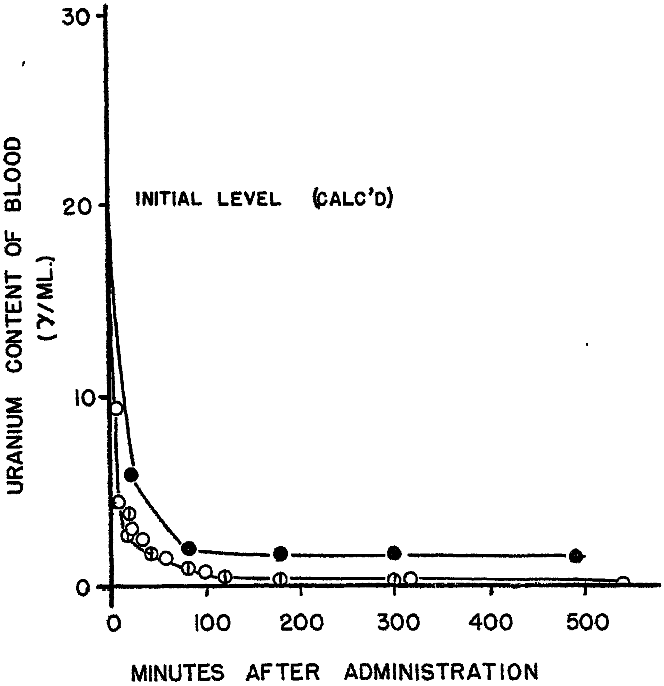

Fig. 1. Disappearance of injected uranium from the blood. ○, specimens from normal rabbits; ●, data from a nephrectomized rabbit.

Table IV contains the summarized results of studies on cats and rabbits. In these animals, nearly one-fifth of the dose was excreted in 4 hours.

It is interesting that a marked difference was observed between the early urinary excretion of the male and the female rats. This was probably a reflection of differences in the amounts of uranium deposited in the skeleton of the two sexes (see below).

Effect of Acid-Base Administration—Work in this laboratory and elsewhere (15, 16) indicated that alkali administration decreased uranium toxicity, as evidenced by mortality. It was not surprising, then, that the simultaneous administration of uranium and sodium bicarbonate resulted in an increase in the amount of uranium excreted. To illustrate the effects of alkali, representative data have been assembled in Table V.

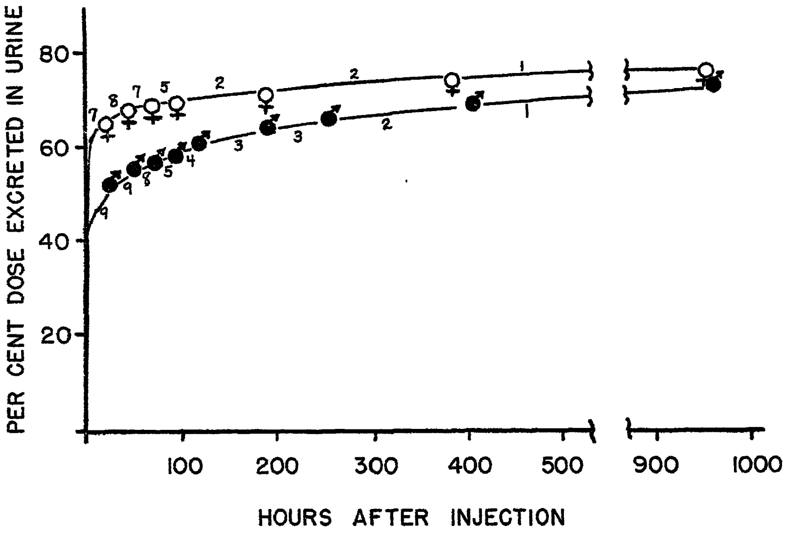

FIG. 2. Averaged urinary excretion by male and female rats. The numbers associated with the curves indicate the number of animals averaged.

TABLE IV

4 Hour Urinary Excretion of Uranium by Individual Cats and Rabbits following Intravenous Injection

Cats		Rabbits	
Dose	Dose in urine	Dose	Dose in urine
mg. U per kg.	*per cent*	*mg. U per kg.*	*per cent*
3.4	17.5	1.7	17.8
3.4	19.0	1.7	8.9
6.2	27.4	1.7	14.2
12.1	18.5	5.6	23.2
Average..........	20.6		16.0

It is apparent that acidifying substances exerted an effect opposed to that of alkaline substances. Rabbits and cats which were given acid-producing diets excreted a much smaller proportion of the dose.

Fecal Excretion—Although the amounts of uranium found in the feces of treated animals were significant, they appear unimportant when compared with the amounts found in the urine. The average total found in the feces

TABLE V

Effect of Acid-Base Administration on 4 Hour Urinary Excretion of Uranium

Species	No. of animals	Diet	Infusion fluid, 0.85%	Systemic acid-base balance	Dose	Dose in urine
					mg. U per kg.	*average per cent*
Cat	1	Normal	$NaHCO_3$	Alkaline	3.4	65
	5	"	NaCl	Normal	3.4–12.1	24
	2	Meat + NH_4Cl	"	Acid	3.4	10
Rabbit	1	Normal	$NaHCO_3$	Alkaline	5.6	71
	4	"	NaCl	Normal	1.7–5.6	16
	3	Oat	"	Acid	1.7–5.6	3.6

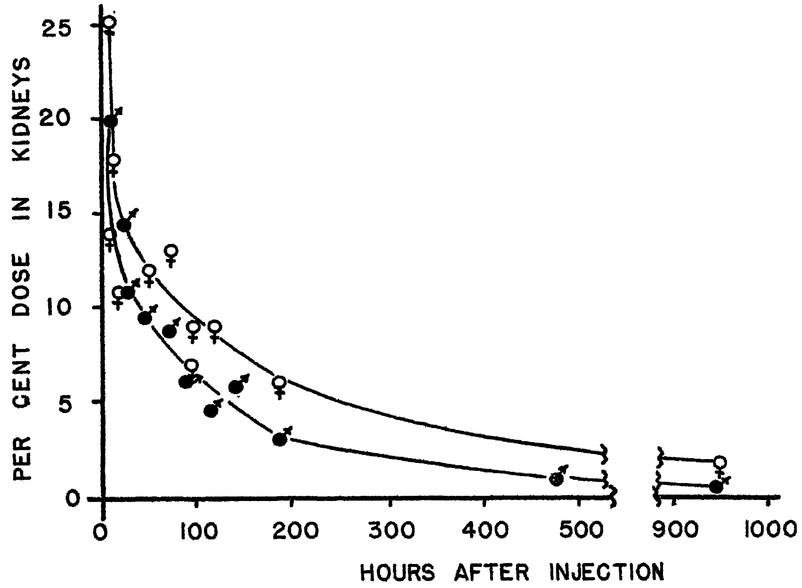

FIG. 3. The relationship between the time after administration and the uranium content of the rat kidney.

of twenty-three rats was only 3.9 per cent of the dose. This average includes fecal collections for periods ranging from 0.75 to 960 hours after administration. There was no correlation between the amount of fecal uranium and the time after injection.

The intestines and contents, removed from the carcass, never contained more than 0.5 per cent of the dose. After 2 days, the quantities found in the intestines were insignificant.

There is good reason to believe that the small amounts in the feces did not represent true excretion, but rather contamination. Two possible sources of contamination were: (*a*) direct contamination; the feces rolled down the sides of the cage funnel which was wet with previously excreted, uranium-rich urine; and (*b*) indirect contamination; a few drops of urine (on the 1st day) clinging to the fur would provide the means by which the rats could obtain several per cent of the dose orally.

TABLE VI

Effect of Acid-Base Relations on Uranium Deposition in Cat Kidney

Diet	Infusion fluid, 0.85%	Dose in kidneys	Dose in urine	Dose in kidney-urine system
		per cent	*per cent*	*per cent*
Normal	NaHCO$_3$	0.8 at 4 hrs.	65	66
"	NaCl	33 " 8 "	20	53
Acid (NH$_4$Cl)	"	48 " 4 "	14	62
" "	"	72 " 8 "	7.9	79

TABLE VII

Uranium Content of Livers of Rats Given Uranyl Nitrate

Time after administration	Uranium concentration in liver	
	Males	Females
hrs.	*γ per gm.*	*γ per gm.*
0.75	0.5	0.6
2.5	0.5	0.3
8	0.3	
12	0.3	0.6
24	0.3	
48	0.4	0.5
72	0.6	0.6
96	0.3	0.5
120	0.3	0.4
288	0.6	0.1
480	0.1	
960	0.1	0.1

Deposition in Kidney—The deposition of uranium in the kidney was very rapid. The highest kidney values, amounting to one-third of the dose, were noted between 0.75 and 2.5 hours after injection. After 40 days, less than 2 per cent of the dose remained. As in the case of urinary excretion, sex difference was observed. The data obtained from rats showing rapid mobilization are presented in Fig. 3.

Data illustrating the effect of acid-base balance on uranium deposition in kidney, presented in Table VI, show an inverse relationship between deposition and systemic alkalinity.

Deposition in Liver—There was practically no deposition in the liver

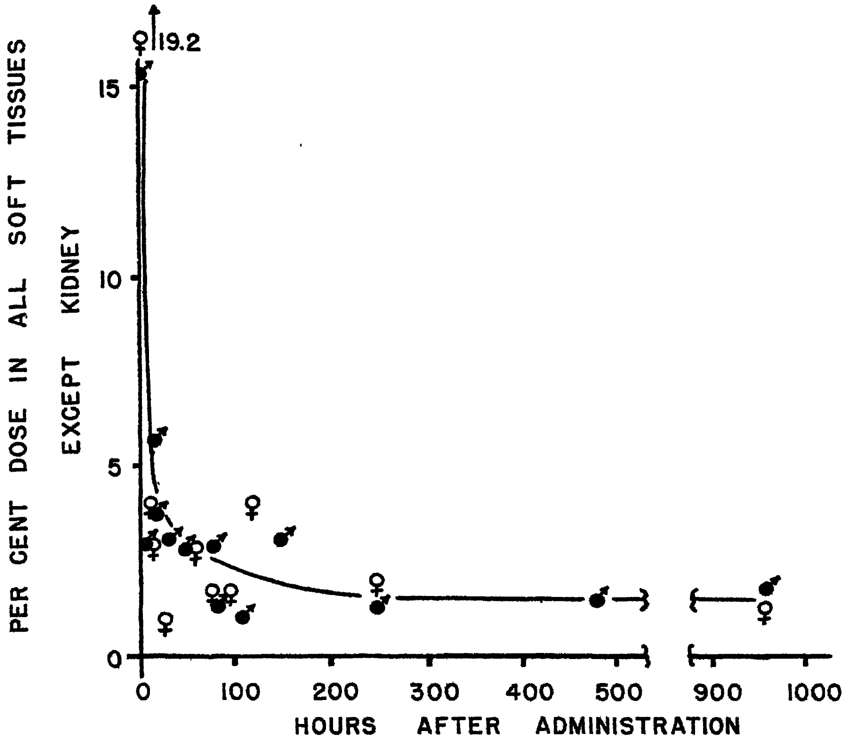

Fig. 4. Disappearance of uranium from the soft tissues

(from 0.1 to 0.9 per cent of the dose), as was indicated by the data from the intravenously injected rats presented in Table VII.

Other species gave similar results. For example, the liver of a dog contained less than 0.2 per cent of the 22.5 mg. of uranyl acetate administered intravenously 3 hours previously.

Deposition in Soft Tissues—Within 45 minutes after administration, all of the soft tissues[2] (excepting kidney) contained *in toto* as much as one-fifth of the uranium administered. At this time there were considerable quantities of uranium in the blood. Later, concurrently with the diminishing blood concentration, the soft tissue content decreased to a very low level (0.2 to 0.3 γ per gm.) and remained low throughout the 40 day time interval. These results are presented graphically in Fig. 4.

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[2] Tissues analyzed separately included bladder, spleen, liver, gonads, heart, lungs, stomach, intestines, skin and hair, and leg muscle. All other non-calcified structures were pooled and analyzed as "soft carcass."

Deposition in Bone—The greatest concentration in bone (20 to 30 per cent of the dose) was noted in 2.5 hours after injection. These data are presented in Fig. 5. Though gradually decreasing thereafter, the quantity found in the skeleton at 40 days accounted for 90 per cent of that remaining in the animal. A consistently greater amount was stored in the bones of males than of females.

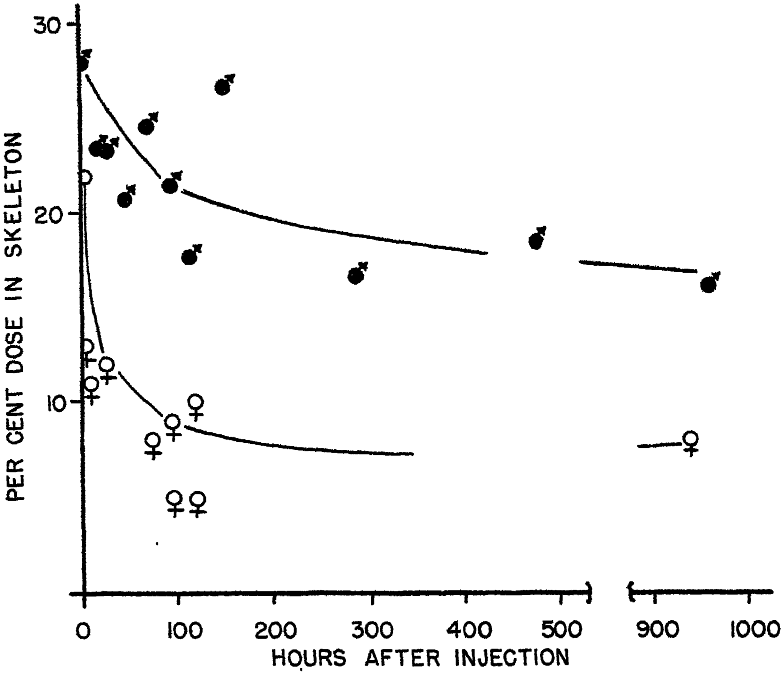

FIG. 5. Data showing a marked sex difference in the deposition of uranium in the rat skeleton.

In view of the fact that liver, spleen, and all soft tissues contained very low concentrations (less than 0.5 γ per gm.), there was little doubt that the uranium found in the skeleton resided in the bone substance and *not* the bone marrow. To confirm this, a femur was removed from a rabbit injected intravenously with uranyl acetate, separated into four portions, and analyzed with the following results:

	Sample weight gm.	Total U content γ
Femur end	5.35	91.2
Marrow from femur end	0.34	0.33
Femur shaft	2.07	25.2
Marrow from femur shaft	1.05	0.66

These data, in spite of the possibility of cross-contamination, clearly showed that marrow does not store significant amounts of injected uranium. As in the case of many other minerals, the end of the femurs, including epiphysis and metaphysis, showed a greater uptake (17 γ per gm.) than did the shaft (12.7 γ per gm.).

DISCUSSION

The rapidity with which uranium left the circulation and appeared in the urine indicates that uranium exists in the animal for the most part in a soluble, diffusible state. Although uranium appeared in the soft tissues shortly after injection, if it penetrated the cells, it was not fixed there to any appreciable extent.

Bone, on the other hand, showed a remarkable affinity for uranium. It is interesting that a consistently greater amount was stored in the bones of males than of females. This suggested that some physiological factor related to sex is involved in bone deposition. However, subsequent studies[3] revealed that the factor in question was age rather than sex. In the experiments reported here, all rats studied weighed 200 ± 5 gm. At this weight, the average male rat of our colony is 9 weeks of age, the average female 16 weeks. The males, therefore, because of a greater rate of bone growth, deposited greater quantities of uranium in the skeleton.

It appeared that the urinary system (kidney and urine collectively) was in competition with the skeletal system for the injected uranium. For example, 100 hours after injection the males showed approximately 20 per cent in the skeleton, 66 per cent in the kidney and urine; the females, 8 per cent in the skeleton, 80 per cent in the kidney and urine. The fact that young animals deposit more uranium in the skeleton,[3] leaving a smaller proportion of the dose to attack the kidney, may account in a large measure for the "resistance" of young animals to the nephrotoxic effects of injected uranium, a fact reported by MacNider (14) and confirmed in this laboratory.

From Table VI it is evident that variations in acid-base balance do not materially alter the distribution between the skeleton and the urinary system. Rather, it determines to what extent the uranium which reaches the kidney is initially fixed in that tissue. Alkali infusion kept kidney fixation below 1 per cent of the dose. Ammonium chloride feeding, on the other hand, increased the deposition in the kidney to as much as 72 per cent. This finding provides a rational basis for the fact that alkali administration diminishes the nephrotoxic effects of injected uranium.

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[3] Neuman, M. W., Neuman, W. F., Main, E., and Mulryan, E., unpublished results.

SUMMARY

Experiments on the distribution and excretion of injected uranium were conducted on rats, rabbits, and cats.

Roughly two-thirds of the dose was excreted rapidly via the urine. As much as one-fifth was found in the skeleton from which it was mobilized slowly. The kidney initially fixed as much as one-fifth of the dose, but the concentration fell rapidly to a very low value in 40 days. All other soft tissues (including blood) contained negligible quantities.

Alkali administration reduced the amount deposited in kidney, increasing proportionately the quantity found in urine. Ammonium chloride feeding exerted an effect opposed to that of alkali administration.

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MERCAPTALS AND MERCAPTOLES OF CYSTEINE

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Our earlier synthesis of djenkolic acid through the condensation of methylene chloride and cysteine (1) in liquid ammonia did not appear to lend itself to the preparation of certain related mercaptals and mercaptoles which we desired. However, the reaction which we have recently described (2) between formaldehyde and cysteine in a strongly acid solution to give djenkolic acid appeared to offer attractive possibilities in this direction. We have now extended this reaction to acetaldehyde, propionaldehyde, and benzaldehyde. When these aldehydes were added to a concentrated solution of cysteine in 6 N hydrochloric acid, the reaction proceeded vigorously and was completed in a few minutes. Some of the products formed insoluble monohydrochlorides and were best purified by recrystallization of this derivative.

We have also found that when acetone and cyclohexanone were caused to react with cysteine under the conditions used for the formation of mercaptals, the corresponding cysteine mercaptoles were obtained.

EXPERIMENTAL

L-*Cysteine Mercaptal of Acetaldehyde*—To a solution of 1.0 gm. of L-cysteine hydrochloride in 2 cc. of 6 N HCl was added 0.65 cc. (0.5 mole) of acetaldehyde, and the solution was allowed to stand at room temperature. After a few seconds the solution became hot and the monohydrochloride of the mercaptal began to crystallize. The mixture was cooled in an ice bath and the solid was collected on a sintered glass filter, washed with cold 6 N HCl, and dried. The yield was 0.70 gm. (65 per cent of the theoretical amount). It was recrystallized several times from 50 per cent ethanol; $[\alpha]_D^{20.5} = -15.0°$ for a 1 per cent solution in 1 N HCl, m.p. 205–230° (with decomposition).[1] The compound gave a negative test for the sulfhydryl and disulfide groups and was not oxidized by iodate.

$C_8H_{17}O_4N_2S_2Cl$.	Calculated.	C 31.52, H 5.59, N 9.20, S 21.02
304.5	Found.	" 31.98, " 5.86, " 9.25, " 21.20

L-*Cysteine Mercaptal of Propionaldehyde*—The preceding experiment was repeated with 0.24 cc. (0.5 mole) of propionaldehyde. The product was obtained and purified in the same manner as the corresponding compound from acetaldehyde. The yield was 0.90 gm. (88 per cent of the theoretical amount); $[\alpha]_D^{20} = +16.0°$ for a 1 per cent solution in 1 N HCl, m.p. 220–222° (with decomposition).

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[1] All melting points are corrected micro melting points.

$C_9H_{19}O_4N_2S_2Cl$. Calculated. C 33.91, H 6.39, N 8.79, S 20.09
318.5 Found. " 34.41, " 6.62, " 8.65, " 20.43

L-*Cysteine Mercaptal of Benzaldehyde*—To a solution of 0.5 gm. of L-cysteine hydrochloride in 1 cc. of 6 N HCl was added 0.33 cc. (0.5 mole) of benzaldehyde and the mixture was shaken at intervals. It became warm and the benzaldehyde gradually dissolved. After standing at room temperature for an hour, the clear solution was diluted with an equal volume of water, the excess benzaldehyde was extracted with ether, and the solution was made neutral to litmus by the addition of 6 N NaOH. The resulting gelatinous precipitate dissolved when the mixture was heated, but reprecipitated as a gel when the solution was cooled. The compound crystallized as small white rosettes after standing overnight at room temperature. The yield was 0.45 gm. (78 per cent of the theoretical amount). The compound was twice recrystallized from water, each time precipitating as a gel which slowly crystallized as small rosettes. The compound gave a negative test for chloride and a negative test for the sulfhydryl and disulfide groups; $[\alpha]_D^{20.5} = +35.0°$ for a 1 per cent solution in 1 N HCl, m.p. 200–220° (with decomposition).

$C_{13}H_{18}O_4N_2S_2$. Calculated. C 47.27, H 4.58, N 8.48, S 19.39
330 Found. " 47.48, " 5.80, " 8.59, " 19.55

L-*Cysteine-Mercaptole of Acetone*—To a solution of 2.5 gm. of L-cysteine hydrochloride in 5 cc. of 6 N HCl were added 1.1 gm. (1 mole) of acetone. After the solution had stood at room temperature overnight, the mercaptole monohydrochloride had crystallized. The precipitate was collected, washed with cold 6 N HCl, and dried. The yield was 2.3 gm. (83 per cent of the theoretical amount). After several recrystallizations from water the compound continued to give a positive test for the sulfhydryl group when treated with sodium nitroprusside.

$C_9H_{19}O_4N_2S_2Cl$. Calculated. C 33.91, H 6.39, N 8.79, S 20.09
318.5 Found. " 34.23, " 6.21, " 8.90, " 20.25

L-*Cysteine Mercaptole of Cyclohexanone*—To a solution of 1.0 gm. of L-cysteine hydrochloride in 2 cc. of 6 N HCl was added 0.34 cc. (0.5 mole) of cyclohexanone. The solution was heated to 70° and was allowed to cool and to stand at room temperature for several days. It was then cooled in an ice bath and was made neutral to litmus by the addition of 6 N NaOH. The mercaptole precipitated as a bulky solid which was collected on a filter, washed with a small amount of cold water, and dried. The yield was 0.20 gm. (20 per cent of the theoretical amount). After two recrystallizations from aqueous ethanol the compound gave a negative test for the sulfhydryl and disulfide groups. The rotation was too small to be measurable for a 1 per cent solution in 1 N HCl; m.p. 245–247° (with decomposition).

$C_{12}H_{22}O_4N_2S_2$.	Calculated.	C 44.58, H 6.81, N 8.68, S 19.82
323	Found.	" 44.68, " 6.93, " 8.37, " 20.07

SUMMARY

Cysteine will condense with simple aldehydes in strongly acid solution to yield cysteine mercaptals. The cysteine mercaptoles of acetone and cyclohexanone were prepared under the same conditions.

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THE REQUIREMENTS OF THE FATTY ACID OXIDASE COMPLEX OF RAT LIVER*

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(Received for publication, January 15, 1948)

In an earlier paper it was shown that suspensions of the washed particulate portion of homogenized rat liver readily oxidized saturated fatty acids in the presence of adenosine triphosphate (ATP), Mg^{++}, and inorganic phosphate (1). The information outlined in that paper allowed a quantitative analysis of the products of enzymatic fatty acid oxidation to be made (2), since such enzyme preparations were essentially free of endogenous respiratory activity. The oxidation of *n*-octanoate in these suspensions was found to proceed according to the equation, $C_7H_{15}COOH + 3O_2 \rightarrow 2CH_3COCH_2COOH + 2H_2O$. However, when the oxidation was allowed to take place in the presence of oxalacetate, the yield of acetoacetate diminished and citrate accumulated, implicating the Krebs tricarboxylic acid cycle in fatty acid oxidation in these enzyme preparations. The latter study also yielded some information as to certain features of the mechanism of fatty acid oxidation, supplementing the isotope studies of Weinhouse and his colleagues (3, 4) and Buchanan *et al.* (5). The gross mechanism of oxidation in the liver of the rat, at least, appears to involve the successive oxidative removal of 2-carbon units from the fatty acid. These units (which are not necessarily identical) may recondense with each other to form acetoacetate or they may react with oxalacetate to form tricarboxylic acids and thus enter into the Krebs cycle.

With this broad outline of the gross mechanism of enzymatic fatty acid oxidation as a guide, attention in this laboratory was again turned to the study of the properties of the fatty acid oxidase complex with the end in mind of dissociating and characterizing separate reaction steps. In the course of attempts to isolate individual reactions of the highly integrated complex of enzymes by variations in the method of preparation, it was found that the enzyme system required several factors in addition to ATP and Mg^{++}. In this paper experiments are described which led to recognition of some additional cofactors in enzymatic fatty acid oxidation and certain properties of the enzyme complex.

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EXPERIMENTAL

Analytical Methods—Acetoacetate was determined colorimetrically by a modification of the method of Greenberg and Lester (6) and citrate by the method of Speck, Moulder, and Evans (7). Octanoate, the fatty acid used as substrate throughout this investigation, was estimated in copper-lime filtrates by a specific method devised in this laboratory (8).

Oxygen uptake was measured in Warburg vessels equipped with alkali and filter paper roll. All manometric measurements were made in air at 30°. In the manometric experiments all components of the reaction medium were present in the main compartment of the Warburg vessels, which were placed in cracked ice. The ice-cold enzyme was added last, and the flasks then placed on manometers and brought to temperature equilibrium in the bath. A 5 to 8 minute temperature equilibration period preceded manometric measurements.

Inorganic and total phosphorus was measured by the Fiske and Subbarow method adapted to photoelectric measurement. Acid-soluble P, phospholipide P, and total nucleic acid P were separated and determined by the method of Schneider (9). Differentiation between pentose- and desoxypentosenucleic acid P was made by the method of Schmidt and Thannhauser (10).

The radioactivity measurements were made on thin layers of aqueous solutions with a standard Geiger-Müller counting tube and conventional scaling apparatus.

Preparative—Adenine nucleotides were prepared as mentioned in a previous paper (1). The diphosphopyridine nucleotide (DPN) used was a product of the Schwarz Laboratories, stated to be 60 per cent pure. Spectrophotometric determination (11) of the purity yielded a value of 51 per cent. The radioactive phosphorus was obtained in the form of $Na_2HP^{32}O_4$, essentially carrier-free, through the United States Atomic Energy Commission.

Two types of enzyme suspension will be referred to throughout this paper, the *saline* suspension and the *water* suspension, and since the manner of preparation of the enzymes is of considerable importance in their requirements for activity, this will be described in detail.

The basic material used in the two types of enzyme preparation is the washed particulate matter of rat liver prepared by a slight modification of the method previously used (1). Iced rat liver, freshly removed from the exsanguinated animal, was homogenized with 2 volumes of ice-cold 0.13 M KCl-0.013 M sodium phosphate buffer, pH 7.8. The homogenate was strained through gauze and diluted with an equal volume of cold KCl-buffer solution. The mixture was centrifuged at 2200*g* in an angle head centrifuge in the cold for 3 minutes. The supernatant was discarded. Fresh, cold

KCl-buffer was added to the residue to equal the original volume of diluted homogenate. The residue was resuspended by shaking and again centrifuged in the cold (2 minutes, 2200*g*). The supernatant was decanted and the residue again taken up in the same volume of KCl-buffer and centrifuged. After decanting the supernatant from the last washing, the residue remaining was used to prepare the two types of enzyme suspension. For the *water* suspension the residue was taken up in cold distilled H_2O to the volume of the original diluted homogenate. For the *saline* suspension the residue was taken up in cold 0.13 M KCl-0.013 M phosphate to the volume of the original diluted homogenate. The *saline* suspension is enzymatically equivalent to the preparation described in a previous paper (1).

Effect of Suspending Enzyme in Water on Activity Requirements—In an earlier paper it was shown that *saline* suspensions of saline-washed particulate matter of rat liver readily oxidized octanoate when supplemented with ATP, Mg^{++}, and phosphate buffer (1). It was also found that if water was used instead of saline for preparation or suspension of the insoluble particles no enzymatic activity could be obtained. Potter has independently confirmed these findings (12). He ascribed the dependence of enzyme activity in the presence of saline in approximately isotonic concentrations to the possibility that the oxidation was taking place only in intact cells, a view which he has since abandoned (13). He also found that when the preparations made by suspending the saline-washed enzyme in water were tested in the presence of extra salt in the Warburg vessel activity could be restored in some preparations but the effect could not be consistently obtained (14).

We have been able to confirm the restoring effect of salt on the *water* suspension of enzyme. However, the restoration of activity was found to be quite erratic, some preparations of enzyme remaining completely inactive.

It was soon found, however, that if low concentrations of certain intermediates of the Krebs tricarboxylic acid cycle were present in the reaction medium in addition to KCl, ATP, Mg^{++}, and phosphate, uniform restoration of activity could be obtained. Furthermore, under these conditions a requirement of cytochrome *c* became much more strongly evident than was the case with the *saline* suspensions of enzyme previously studied (see also Potter (12)). In Table I are shown the data collected from experiments designed to compare the requirements for activity of the *saline* suspension of enzyme and the *water* suspension of enzyme. The *saline* suspension of the enzyme does not require the addition of KCl, malate, or cytochrome *c* for strong activity; these substances do not accelerate the oxidation greatly when added to the system. However, in the *water* suspension of enzyme the absolute nature of the requirement for neutral salt and malate is strikingly evident, as well as the substantial requirement for the presence of cytochrome *c*. The criteria of fatty acid oxidase activity were measurements of oxygen uptake, octanoate disappearance, and acetoacetate formation.

Treatment of the saline-washed particulate matter of rat liver with water, then, reveals that the enzymatic oxidation of octanoate to acetoacetate requires the presence of ATP, Mg^{++}, a neutral salt such as KCl, a small amount of malate, and cytochrome *c*. If the saline-washed particles are suspended in saline, however, they show an absolute requirement for only ATP and Mg^{++}.

TABLE I

Comparison of Requirements of Saline and Water Suspensions of Enzyme

In the *saline* suspension test, the Warburg vessels contained 1.7 ml. of *saline* suspension (added last), 0.25 ml. of MgSO$_4$ (0.005 M),* 0.25 ml. of KCl (0.05 M), 0.50 ml. of phosphate buffer, pH 7.4 (0.01 M), 0.50 ml. of ATP (0.0005 M), 0.25 ml. of sodium octanoate (0.001 M, total 5.0 micromoles), 0.50 ml. of cytochrome *c* (1×10^{-5} M), 0.25 ml. of sodium *l*-malate (0.0003 M), and water to make 5.0 ml. When the components were omitted, an equal volume of water was substituted. Time, 40 minutes. In the *water* suspension test, the system was arranged exactly as in the *saline* suspension test. Time, 55 minutes.

		Oxygen uptake	Octanoate removed	Acetoacetate formed
		micromoles	*micromoles*	*micromoles*
Saline suspension	Complete system	16.7	4.8	9.2
	Octanoate omitted	2.0		0.3
	Malate omitted	14.0	4.9	10.4
	KCl omitted	14.8	4.8	9.1
	MgSO$_4$ omitted	4.3	1.1	3.4
	ATP omitted	0.6	0.4	0.3
	Cytochrome *c* omitted	14.2	4.4	8.1
Water suspension	Complete system	13.6	4.5	7.9
	Octanoate omitted	3.1		0.1
	Malate omitted	1.0	0.1	0.2
	KCl omitted	1.7	0.1	0.0
	MgSO$_4$ omitted	4.3	1.6	3.2
	ATP omitted	0.2	0.0	0.2
	Cytochrome *c* omitted	3.6	2.0	2.0

* The figures in parentheses refer to final concentration of each component in the complete reaction medium. This notation is used in all the tables in this paper.

The effects and possible functions of some of the components required for fatty acid oxidase activity will now be considered.

Requirement of Adenine Nucleotides—Previous work indicated that in the *saline* suspension of washed liver particles ATP was required for fatty acid oxidation, whereas adenosine diphosphate (ADP) and muscle adenylic acid were ineffective (1). However, it has since been found that under certain conditions the three nucleotides are equally effective, both in the *saline* suspension and in the *water* suspension. In the earlier study the enzyme was incubated with all components of the system except substrate for some 5 minutes (temperature equilibration period), followed by addition of substrate from the side arm. Under these conditions only ATP was found effective. However, if *all* components including fatty acid substrate are initially present in the main compartment of the chilled Warburg vessel, ice-cold enzyme being added last, and the contents then incubated, all three nucleotides are equally effective in activating the oxidation. Study of the rates of dephosphorylation of the nucleotides by the enzyme revealed that all three nucleotides undergo rapid dephosphorylation. In the 5 minute period of incubation before substrate was added (in the earlier experiments) extensive dephosphorylation and deamination of the nucleotides were obviously taking place. However, since ATP has three phosphate groups which must be consecutively removed by phosphatase action to form adenosine, which is inactive, it is possible that the apparently specific effectiveness of ATP in the earlier experiments was due to sparing of some of the nucleotide by the presence of the additional phosphate groups. After fatty acid oxidation has once begun, phosphorylations coupled to the oxidation continually regenerate ATP or ADP from adenylic acid, as experiments below will show.

TABLE II

Requirement of Adenine Nucleotides

The main compartment of the Warburg vessels contained 0.90 ml. of *water* suspension of enzyme, 0.15 ml. of $MgSO_4$ (0.005 M), 0.15 ml. of KCl (0.05 M), 0.30 ml. of nucleotide, or adenosine, or adenine, 0.30 ml. of phosphate buffer, pH 7.4 (0.01 M), 0.30 ml. of cytochrome *c* (10^{-5} M), 0.15 ml. of *l*-malate (0.0005 M), 0.15 ml. of sodium octanoate (0.001 M), and water to make 3.0 ml. Time, 40 minutes.

	Oxygen uptake	Acetoacetate formed
	micromoles	*micromoles*
0.001 M ATP	8.1	2.5
0.001 " ADP	8.4	3.0
0.001 " muscle adenylic acid	9.3	2.6
0.001 " yeast " "	1.4	0.0
0.001 " adenosine	0.4	0.1
0.001 " adenine	0.5	0.1
0.001 " DPN (51% pure)	8.9	2.4
0.0001 " " (51% ")	3.2	0.7
No addition	0.7	0.0

In Table II are shown data obtained with the *water* suspension of enzyme, indicating the effectiveness of the three nucleotides and the ineffectiveness of yeast adenylic acid, adenosine, and adenine under the same conditions.

In the course of these experiments it was found that preparations of DPN of approximately 50 per cent purity can replace the adenine nucleotides in the system. Whether this is due to the presence of adenine nucleotides as impurities in the DPN preparations or to the enzymatic scission of DPN to form an adenine mononucleotide is not clear. This effect of DPN may be related to the occasional effectiveness of DPN in restoring activity of aged fatty acid oxidase preparations (1).

Although all three adenine mononucleotides are effective in activating the oxidation of octanoate, it may not be safely concluded that adenylic acid, as the least common denominator, is the immediately required nucleotide, since data reported below show that the nucleotides undergo active aerobic phosphorylation. It is quite possible that a mixture of all three nucleotides may be present during the oxidation following the addition of any one nucleotide, as a resultant of phosphorylation and dephosphorylation reactions.

Since earlier work pointed to ATP as the active nucleotide, a tentative hypothesis had been set up at that time concerning the mechanism of action of ATP which involved an obligatory phosphorylation of fatty acid by the ATP prior to oxidation (15, 16). This hypothesis gained some experimental support when it was found that synthetic acyl phosphates of higher fatty acids (17) readily donated phosphate enzymatically to adenylic acid in crude homogenates of rat liver.[1] Subsequently, the development of the washed enzyme suspension, free of endogenous activity (1), allowed a more critical test of the synthetic acyl phosphates as intermediates in fatty acid oxidation. It was found that the washed liver suspensions, in contrast to the crude liver homogenate, were not capable of catalyzing the transphosphorylation reaction observed in the crude homogenate. Furthermore, the synthetic acyl phosphates show no special activities in either the *saline* suspension or *water* suspension of the washed enzyme not shown by free fatty acid salts. The transphosphorylation reaction observed in crude liver homogenates, therefore, appears not to be concerned in fatty acid oxidation. It may, however, be concerned in other metabolic reactions of fatty acids.

Requirement of Inorganic Phosphate—In Table III are shown results of an experiment demonstrating that the presence of inorganic phosphate is required for fatty acid oxidation. Since the reaction medium was ordinarily buffered with inorganic phosphate, it was necessary to substitute another buffer. Tris(hydroxymethyl)aminomethane-HCl buffer (18) proved to be ideal for the system. It can be seen that omission of inorganic phosphate results in greatly decreased activity. The small amount of inorganic phosphate already present in the enzyme preparations probably is responsible for this minimal activity. Raising the concentration of inorganic phosphate causes concomitant increases in the rate of oxidation until a plateau is reached at a concentration of approximately 0.0005 M inorganic phosphate. Obviously, the system is capable of functioning maximally with rather low concentrations of inorganic phosphate.

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[1] Lehninger, A. L., unpublished experiments.

The requirement of inorganic phosphate for activity of the fatty acid oxidase system is probably related to the ability of the system to cause esterification of inorganic phosphate coupled to the oxidation of fatty acid. In Table IV are shown supporting data. It can be seen that in the complete system the oxidation of fatty acid maintains the level of acid-labile esterified phosphate (P liberated by 7 minutes hydrolysis at 100° in 1 N H_2SO_4). When fatty acid is omitted, there is some maintenance of the esterified phosphate owing to the oxidation of the small amounts of malate present. When both substrates are omitted, there is no oxidation and no significant maintenance of esterified phosphate. The maintenance of esterified phosphate in the complete system is probably due to the fact that the rate of esterification of inorganic phosphate coupled to the oxidation of fatty acid approaches the rate of dephosphorylation of phosphate esters by phosphatases, the result being maintenance of the level of esterified phosphate. In the absence of oxidations no esterification takes place and the phosphatases quickly dephosphorylate the adenine nucleotides and other

TABLE III

Inorganic Phosphate Requirement

The Warburg vessels contained 0.90 ml. of *water* suspension of enzyme, 0.15 ml. of $MgSO_4$ (0.005 M), 0.15 ml. of KCl (0.05 M), 0.30 ml. of cytochrome *c* (10^{-5} M), 0.30 ml. of sodium adenylate (0.001 M), 0.15 ml. of octanoate (0.001 M), 0.30 ml. of tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.5, 0.15 ml. of malate (0.0005 M), concentrations of inorganic phosphate indicated below, and water to make 3.0 ml. Time, 30 minutes.

Inorganic phosphate		O_2 uptake	Acetoacetate formed
Added	Present*		
M	*M*	*micromoles*	*micromoles*
0	0.0002	1.3	0.3
0.0001	0.0003	2.7	1.4
0.0002	0.0004	5.9	2.5
0.0003	0.0005	7.1	3.8
0.0004	0.0006	7.2	3.6
0.001	0.0012	7.6	3.9
0.010	0.010	7.4	3.8

* The enzyme preparation contained sufficient inorganic phosphate to give a concentration of about 0.0002 M.

phosphate esters present. Several attempts have been made to demonstrate a *net* synthesis of newly esterified phosphate by using various phosphate acceptors such as creatine, glucose, glucose-6-phosphate, etc., to trap esterified phosphate. However, the necessary transphosphorylases appear not to be present in these enzyme suspensions.

In order to demonstrate unequivocally that the maintenance of esterified phosphate shown in Table IV is actually the resultant of the oxidation-coupled synthesis of new phosphate linkages and phosphatase action, we have allowed the oxidation of octanoate to take place in a medium containing inorganic phosphate labeled with P^{32}. After completion of the incubation, trichloroacetic acid filtrates were prepared and the radioactivity of the esterified fraction of the acid-soluble phosphorus was measured after removal of inorganic P^{32} by dilution with carrier phosphate and separation with magnesia mixture. The results, shown in Table V, clearly demonstrate substantial incorporation of inorganic P^{32} into the esterified fraction coupled to fatty acid oxidation. When octanoate was omitted, some esterification took place owing to the oxidation of the malate present. When both octanoate and malate were omitted, very little incorporation occurred. These experiments, therefore, demonstrate that at least part of the free energy released during fatty acid oxidation is recovered by coupled synthesis of new phosphate bonds.

TABLE IV

Maintenance of Level of Esterified Phosphate by Octanoate Oxidation

The Warburg vessels contained 0.90 ml. of *water* suspension of enzyme, 0.15 ml. of $MgSO_4$ (0.005 M), 0.15 ml. of KCl (0.05 M), 0.60 ml. ATP (= 227 γ of 7 minute-hydrolyzable P), 0.30 ml. of cytochrome *c* (1×10^{-5} M), 0.30 ml. of tris(hydroxymethyl)-aminomethane-HCl buffer, pH 7.4 (0.01 M), 0.15 ml. of octanoate (0.001 M), 0.15 ml. of *l*-malate (0.0005 M), and H_2O to make 3.0 ml. When components were omitted, H_2O was substituted. The side arm contained 0.3 ml. of 3 N HCl; tipped to stop reaction at specified times.

	Time	O_2 uptake	Acetoacetate formed	Inorganic P	7 min. hydrolyzable P
	min.	*micromoles*	*micromoles*	γ	γ
Complete system	0			113	256
" "	25	6.5	3.0	148	248
Octanoate omitted	25	1.2	0.1	278	105
l-Malate "	25	1.1	0.3	423	65
Both octanoate and *l*-malate omitted	25	0.3	0.0	445	35

Malate Requirement—Since the addition of small amounts of certain intermediates of the Krebs cycle to the reaction medium was found to be necessary for complete restoration of the activity of the *water* suspension of enzyme, this effect was studied from the standpoint of specificity. As has been mentioned before, not all preparations require the addition of such a compound for fatty acid oxidase activity, presumably because small but sufficient amounts of such substances are already present in some of the enzyme preparations. In order to study this effect quantitatively, it was necessary to use only enzyme preparations which showed no fatty acid oxidase activity unless the activator of the Krebs cycle was present. Such preparations were consistently obtained by the simple expedient of making the enzyme preparation with only one-half of the tissue concentration ordinarily used so that the end-product, the *water* suspension of enzyme, contained only about one-half the usual enzyme concentration and was somewhat more thoroughly washed. These preparations were used in the experiments reported in Table VI. In Experiments I and II a variety of compounds were tested for their ability to activate octanoate oxidation, all other known requirements being supplied at optimal concentration. The criteria used were oxygen uptake and octanoate utilization. In the absence of added activating compound, no octanoate oxidation took place. The compounds found to be effective in activating the oxidation were *cis*-aconitate, citrate, malate, oxalacetate, α-ketoglutarate, succinate, and fumarate. However, lactate, pyruvate, *dl*-β-hydroxybutyrate, acetate, ascorbate, and *dl*-phosphomalate were found to be inactive when tested at the same concentration (0.0005 M). Acetaldehyde showed a slight activat-

TABLE V

Incorporation of Inorganic Phosphate Labeled with P^{32} into Esterified Phosphate Fraction Coupled to Fatty Acid Oxidation

The main compartment of the Warburg vessels contained 0.60 ml. of diluted *water* suspension of enzyme, 0.10 ml. of KCl (0.05 M), 0.10 ml. of $MgSO_4$ (0.005 M), 0.20 ml. of tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.5 (0.01 M), 0.40 ml. of ATP (0.001 M), 0.20 ml. of cytochrome *c* (1 × 10^{-5} M), 0.10 ml. of malate (0.0005 M), 0.10 ml. of octanoate (0.001 M), 0.10 ml. of inorganic phosphate containing P^{32} (363,300 counts per minute, total), and H_2O to make 2.0 ml. Incubated at 30° for 8 minutes; taps closed and manometric measurements made for the following 17 minutes.

	O_2 uptake	Inorganic P	Esterified P	Specific activity of esterified P
	micromoles	γ	γ	*counts per min. per γ esterified P*
Complete system (0 time)		120	194	12
"	3.2	166	157	1010
Octanoate omitted	0.3	223	93	590
" and malate omitted	0.2	246	54	167

ing effect. Octanoate oxidation, therefore, requires the presence of some intermediate of the Krebs tricarboxylic acid cycle other than pyruvate.

Further experiments disclosed that the rate of oxidation of isocitrate to α-ketoglutarate is the slowest oxidative reaction of the Krebs cycle in these diluted enzyme preparations. Citrate and *cis*-aconitate when tested for their activating effect on octanoate oxidation show a considerable lag period before extensive removal of octanoate occurs. No lag period is observed with α-ketoglutarate, succinate, malate, or oxalacetate under the same conditions. Tentatively, the presence of citrate or the oxidation of citrate to α-ketoglutarate may be excluded as the activating factor.

TABLE VI

Requirement of Intermediates of Krebs Cycle

The main compartment of the Warburg vessels contained 1.5 ml. of dilute *water* suspension (see the text), 0.25 ml. of $MgSO_4$ (0.005 M), 0.25 ml. of KCl (0.05 M), 0.50 ml. of ATP (0.0006 M), 0.50 ml. of phosphate buffer, pH 7.4 (0.01 M), 0.50 ml. of cytochrome *c* (1×10^{-5} M), 0.25 ml. of octanoate (0.001 M), 0.25 ml. of activating substrate (0.0005 M), and water to make 5.0 ml. In Experiment III, 0.01 M malonate was also present. Time, 60 minutes in each experiment.

Experiment No.	Activator	O_2 uptake	Octanoate utilized
		micromoles	*micromoles*
I	None	0.1	0.2
	l-Malate	18.8	4.9
	Oxalacetate	17.6	4.9
	dl-β-Hydroxybutyrate	1.7	0.2
	Pyruvate	2.5	0.3
	Acetaldehyde	4.0	0.6
	dl-Phosphomalate	1.0	0.1
	Acetate	1.2	0.2
II	None	0.9	0.4
	cis-Aconitate	8.2	2.4
	Citrate	8.0	2.7
	α-Ketoglutarate	10.1	3.7
	Succinate	10.2	3.9
	Fumarate	10.0	3.9
	Lactate	1.2	0.6
	Ascorbate	1.1	0.3
III	None	0.0	0.2
	α-Ketoglutarate	1.3	0.4
	Succinate	0.1	0.1
	l-Malate	4.2	1.2
	Oxalacetate	4.4	1.5

Further localization of the activating reaction was achieved by the use of malonate as an inhibitor of succinic dehydrogenase (Table VI, Experiment

III). Although malonate also inhibited fatty acid oxidation strongly in these experiments,[2] it was found that citrate, α-ketoglutarate, and succinate failed to activate octanoate oxidation in the presence of malonate, whereas malate and oxalacetate were still capable of activating the oxidation. The activating effect of the intermediates of the Krebs cycle may therefore be tentatively localized to some effect of the presence of malate or oxalacetate.

Previous work has shown that intermediates of the Krebs cycle, when added to the *saline* suspension of enzyme, cause a decrease in yield of acetoacetate from the theoretical 2 moles per mole of octanoate oxidized, some of the fatty acid carbon being diverted into the formation of citrate via a 2-carbon intermediate (2). The present study indicates that malate or oxalacetate is required in the water-treated enzyme for the oxidation of octanoate to acetoacetate. The latter finding implies that malate actually has two distinct functions in the oxidase system, one to activate the oxidation of octanoate to acetoacetate (or the 2-carbon intermediate), and the other to react with 2-carbon units to form citrate and thus cause fatty acid oxidation products to enter the Krebs cycle. The results of the experiment presented in Table VII illustrate the dual function of malate in the fatty acid oxidase system. In this experiment the *water* suspension of enzyme was allowed to act upon octanoate in the presence of varying concentrations of malate. Measurements of oxygen uptake, octanoate utilization, acetoacetate formation, and citrate formation were made. In the absence of added malate there was no oxidation of octanoate. With 0.0001 M malate suboptimal oxidation of octanoate occurred. At 0.0005 M malate concentration, maximal octanoate disappearance occurred. Somewhat less than the theoretical yield of 2 moles of acetoacetate was formed per mole of octanoate disappearing. Raising the malate concentration from 0.0005 M to as high as 0.02 M caused no increase in the octanoate utilization, but produced a great decrease in yield of acetoacetate and a great increase in yield of citrate. Therefore, it is clear that a small amount of malate is required to initiate oxidation of the fatty acid; in higher concentrations malate has the additional effect of diverting fatty acid carbon from acetoacetate formation into citrate formation, probably by furnishing a greater supply of oxalacetate for condensation to form tricarboxylic acid.

The amounts of malate required for the primary phase, *i.e.* catalysis of oxidation of octanoate to the stage of acetoacetate or the hypothetical 2-carbon fragment, are quite low and, compared to the amounts of octanoate utilized, are catalytic in magnitude. For instance, in Table VII, at a malate concentration of 0.0001 M (total malate added, 0.5 micromole) 1.5 micromoles of octanoate disappeared. The addition of 1 molecule of malate, which itself is undergoing continuous oxidative removal, therefore sufficed to cause the disappearance of 3 molecules of octanoate. When malate was omitted, no significant disappearance of octanoate occurred.

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[2] It has been found that malonate has varying inhibitory effects on the fatty acid oxidase system, depending on the strain of rat used for preparation of the enzyme. Preparations made from livers of Sprague-Dawley rats are not inhibited more than about 25 per cent by 0.01 M malonate. In an earlier paper (2) all experiments were done with this strain in the presence of 0.01 M malonate to minimize endogenous respiration. However, preparations made from livers of other strains of rats are much more sensitive. A heterogeneous stock colony used in this work showed great sensitivity; 0.002 M malonate produced 50 to 75 per cent inhibition of octanoate oxidation.

Since the *saline* suspension of enzyme does not require the addition of catalytic amounts of malate or oxalacetate, it is possible that the activating intermediates are actually present in the particulate material but are "released" from the particles by treatment with water, causing dilution beyond an active concentration. This explanation may also account for the necessity of adding cytochrome *c* after water treatment, since the *saline* suspension does not require the addition of cytochrome *c* for strong activity. If this particulate material represents some subcellular structure preexisting in the cell, it is possible that subjecting the particles to hypotonic solutions causes a change in permeability of a limiting membrane with loss of solutes from within the structure. Certain intracellular inclusions are known to be sensitive to changes in osmotic pressure (19).

TABLE VII

Effect of Malate Concentration on Products of Octanoate Oxidation

The Warburg vessels contained 1.50 ml. of dilute *water* suspension, 0.25 ml. of KCl (0.05 M), 0.25 ml. of $MgSO_4$ (0.005 M), 0.50 ml. of ATP (0.0005 M), 0.50 ml. of cytochrome *c* (1×10^{-5} M), 0.25 ml. of octanoate (0.001 M = 5 micromoles), 0.25 ml. of malate in the concentrations listed, 0.50 ml. of phosphate buffer, pH 7.4 (0.01 M), and water to make 5.0 ml. Time, 55 minutes.

Malate concentration	Octanoate	O_2 uptake	Octanoate utilized	Acetoacetate formed	Citrate formed
M		*micromoles*	*micromoles*	*micromoles*	*micromoles*
0	+	0.0	0.2	0.0	0.3
0.0001	+	2.3	1.5	2.7	0.4
0.0005	–	4.4		0.3	0.6
0.0005	+	10.4	3.0	4.3	1.2
0.002	–	5.8		0.0	1.0
0.002	+	12.1	3.1	3.3	2.8
0.008	+	13.2	3.1	1.8	3.4
0.02	–	6.6		0.1	1.2
0.02	+	14.0	3.1	0.8	5.6

Effect of Salts—One of the striking properties of the *water* suspension of enzyme is the complete dependence on KCl for activity. We have investigated the effect of variations in the activity of the enzyme caused by variation in KCl concentration and also the activity of a variety of other compounds substituted for KCl. In Fig. 1 are shown the effects of varying KCl concentrations on the oxidase activity and comparable experiments in which NaCl, sucrose, glycine, and urea were substituted for KCl. The criteria of activity were oxygen uptake and acetoacetate formation. Since a number of different enzyme suspensions were used, a standard system containing 0.05 M KCl was assumed to be 100 per cent active and all activities were expressed in per cent activity of this standard system. In all cases oxygen uptake and acetoacetate formation were parallel.

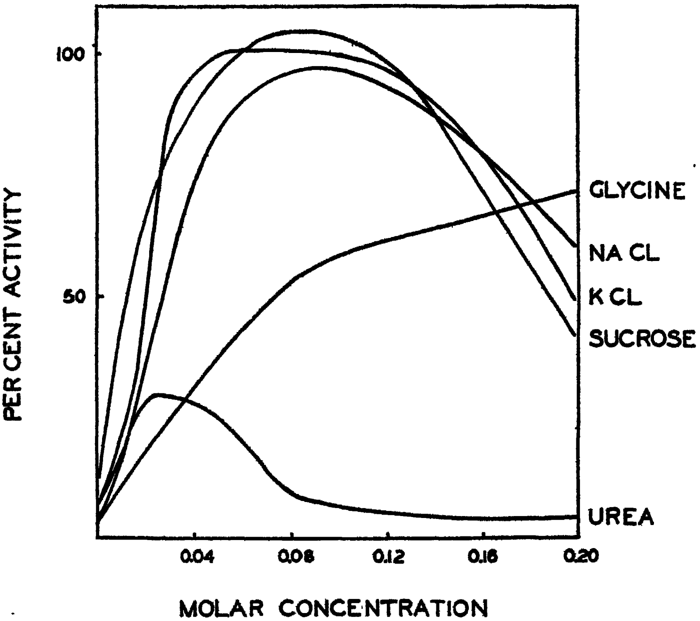

Fig. 1. Effect of salts and non-electrolytes on fatty acid oxidase activity. The main compartment of the Warburg vessel contained 0.90 ml. of *water* suspension of enzyme, 0.15 ml. of $MgSO_4$ (0.005 M), 0.60 ml. of ATP (0.001 M), 0.30 ml. of cytochrome *c*, 0.30 ml. of phosphate buffer, pH 7.4 (0.01 M), 0.15 ml. of malate (0.0005 M), 0.15 ml. of octanoate (0.001 M), various compounds in final concentrations over the range indicated in the figure, and water to make 3.0 ml. The time varied between 50 and 70 minutes in the different experiments. 100 per cent activity represents acetoacetate formation from octanoate in a system containing 0.05 M KCl.

It can be seen from Fig. 1 that KCl was maximally effective in a wide range of concentration from 0.05 M to 0.12 M. Half maximal activation was shown by a concentration of about 0.02 M. Sodium chloride appears to be equally effective in substituting for KCl. Surprisingly, sucrose showed a nearly identical effect. Glycine, which is not oxidized in these preparations, was also active, although much higher molar concentrations (0.20 M) were necessary to achieve maximal activation. Urea showed some effectiveness at 0.02 M, none at higher concentrations.

Other findings may be summarized. Glucose, fructose, and xylose showed strong activating ability, whereas glycerol, acetone, acetamide, glycogen, and ethylene glycol were inactive. Calcium, magnesium, and barium salts were ineffective. Lithium chloride was as effective as KCl or NaCl. Alanine was somewhat less effective than glycine.

The requirement of salts (or solutes) for oxidative activity is not limited to fatty acid oxidation. The oxidation of malate and the oxidation of pyruvate to acetoacetate by the *water* suspension of enzyme are also completely dependent on the presence of a solute (see Table VIII).

TABLE VIII

Salt Requirement in Oxidation of Malate and Pyruvate

The Warburg vessels contained 1.0 ml. of *water* suspension of enzyme, $MgSO_4$, KCl, phosphate buffer, cytochrome *c*, and ATP in the same concentrations as in the standard system of Table II plus pyruvate (0.01 M) or malate (0.01 M) (octanoate was not present). When KCl was omitted, water was substituted. Total volume, 3.0 ml. Time, 40 minutes.

Substrate	KCl	O_2 uptake	Acetoacetate formed
		micromoles	*micromoles*
Pyruvate	+	3.2	3.1
"	−	0.1	0.0
l-Malate	+	4.8	
"	−	0.1	

Potter has presented some evidence that potassium ions have a specific effect in activating the fatty acid oxidase (12). We have found no essential difference between potassium and sodium chloride as activators of the system. However, since KCl was used in the preparation of the enzyme, it appeared that sufficient potassium ions were present in the enzyme to cause maximal activation. In order to test this, a sample of *water* suspension of enzyme was prepared with 0.13 M NaCl-0.013 M phosphate buffer as the homogenizing and washing medium instead of the KCl-phosphate mixture. The washed residue was taken up in water and tested in the presence of 0.05 M NaCl and 0.05 M KCl in separate flasks. Both showed the same activity. Analysis of the enzyme prepared with potassium-free reagents showed that 1.0 ml. of enzyme contained 0.0023 milliequivalent of total potassium. If potassium is a specific requirement of the oxidase, its effect is maximal at concentrations of approximately 0.0007 M in the test system.

During the course of these experiments it was observed that the complete reaction medium, containing the *water* suspension of enzyme and all components required for activity, presented a more opaque or turbid appearance in transmitted light than did the same medium with the activating salt omitted. This observation led to experiments on the *water* suspension of enzyme to determine what physical changes could be brought about by the addition of salts, non-electrolytes, etc. It was found that when KCl was added to the *water* suspension of enzyme to make a concentration of between 0.07 and 0.15 M there was an immediate change from a relatively transparent to an opaque appearance. On centrifuging, a cream-colored precipitate separated sharply, leaving an almost clear supernatant containing hemoglobin. On the other hand, the untreated *water* suspension of enzyme when centrifuged under the same conditions, yielded a small amount of reddish brown precipitate which did not separate sharply from the very turbid supernatant. The effect of KCl on the *water* suspension of enzyme is to bring about a flocculated condition of a component of the suspension. Since the presence of neutral salt is required for fatty acid oxidase activity, this flocculated condition of the enzyme suspension appeared to be associated with the enzyme activity and the dispersed condition (absence of salt) with inactivity.

That this assumption is valid was proved by the following experiment in which the material flocculated from *water* suspension of the enzyme by KCl was separated and tested for enzyme activity. 9 ml. of *water* suspension of enzyme were treated with 1.0 ml. of 1.0 M KCl, allowed to stand for 5 minutes in an ice bath, and then centrifuged in the cold. The slightly turbid, pink supernatant was decanted, leaving a well packed, cream-colored precipitate which was taken up in ice-cold distilled water to make 10.0 ml. Samples of the original *water* suspension, the resuspended KCl-precipitated material, and the supernatant from the latter were assayed for fatty acid oxidase activity. The results are given in Table IX, Experiment I. It is obvious that the material flocculated by KCl contains all of the oxidase activity of the original water suspension, the supernatant having no activity. In another experiment, the salt-flocculated enzyme was washed with another portion of 0.1 M KCl and then resuspended in water and tested for activity to determine whether traces of the original supernatant were necessary for activity. This precipitated, washed enzyme was found to be strongly active (Experiment II, Table IX). The data show that this salt-precipitated enzyme also requires neutral salt for activity, as well as other components known to be required by the *water* suspension of washed liver residue.

Analysis of the *water* suspension and the KCl-precipitated enzyme obtained from the *water* suspension for dry weight, total nitrogen, total phosphorus, phospholipide phosphorus, and nucleic acid phosphorus revealed that the salt-precipitated material contained about 77 per cent of the dry weight, 72 per cent of the total nitrogen, 77 per cent of the total phosphorus, 75 per cent of the phospholipide P, and 95 per cent of the nucleic acid P of the original *water* suspension of enzyme. The salt-precipitated enzyme was nearly devoid of cytochrome *c*, the latter remaining in the supernatant. Analysis (10) of the salt-precipitated enzyme revealed that the nucleic acid present was a mixture of the pentose and desoxypentose types in about equal proportions.

TABLE IX

Fatty Acid Oxidase Activity of Material Precipitated by KCl from Water Suspension

The main compartment of the Warburg vessels contained 0.90 ml. of enzyme suspension as indicated below, 0.15 ml. of $MgSO_4$ (0.005 M), 0.15 ml. of KCl (0.05 M), 0.30 ml. of ATP (0.0005 M), 0.30 ml. of phosphate buffer (0.01 M), 0.30 ml. of cytochrome *c* (1×10^{-5} M), 0.15 ml. of sodium octanoate (0.001 M), 0.15 ml. of malate (0.0005 M), and water to make 3.0 ml. When KCl was introduced with the enzyme (supernatant), the KCl concentration added to the flask was compensated to give a final concentration of 0.05 M. Time, 50 minutes in Experiment I, 65 minutes in Experiment II.

Experiment No.	Enzyme source	Oxygen uptake	Acetoacetate formed
		micromoles	*micromoles*
I	*Water* suspension, complete system	10.8	
	Same, octanoate omitted	3.2	
	KCl-pptd. material, complete system	10.8	
	Supernatant, complete system	0.0	
	KCl-ppt. + supernatant, complete system	10.3	
II	KCl-pptd. enzyme, washed once with 0.1 M KCl, complete system	8.3	3.5
	Same, octanoate omitted	2.6	0.0
	Malate omitted	1.3	0.9
	Cytochrome *c* omitted	0.8	0.3
	KCl omitted	1.1	0.1
	ATP "	0.0	0.0
	Mg^{++} "	2.9	0.4

NaCl, LiCl, and sucrose also cause the flocculation of the enzyme complex from the *water* suspension.

DISCUSSION

In earlier papers (16, 1) the senior author elaborated a working hypothesis for the observation of Leloir and Muñoz (20, 21) that the presence of adenylic acid and fumarate was required to demonstrate the oxidation of butyrate by a washed preparation of guinea pig liver. It was proposed that these two factors were necessary to cause the enzymatic generation of adenosine triphosphate from adenylic acid coupled to the oxidation of fumarate and that ATP was actually the immediately necessary cofactor for fatty acid oxidation. As it happened, experiments based on this hypothesis proved highly successful in subsequent work (1), although the data presented in this paper make that hypothesis somewhat less tenable. Both adenine nucleotide and malate or oxalacetate appear to be required for enzymatic fatty acid oxidation; ATP is not capable of substituting for the two required compounds. This requirement of the system became obvious only after some manipulations of the enzyme, which resemble those employed by Leloir and Muñoz. The latter investigators apparently did not recognize fully the requirement for salts or non-electrolytes demonstrated in this paper; this factor may possibly account for the erratic activity of their enzyme preparations.

The finding that an intermediate of the Krebs cycle is required in addition to adenine nucleotide makes possible a closer correlation of the properties of the enzyme from guinea pig liver studied by Leloir and Muñoz, the fatty acid oxidase of heart muscle (22), and the oxidase system of rat liver studied here, all of which require both adenine nucleotide and malate or oxalacetate.

The mechanism of action of the required intermediate of the Krebs cycle, which our experiments tentatively indicate to be either malate or oxalacetate, is obscure and considerable work on this phase of the oxidation has not yielded fully definitive results. However, the data presented clearly indicate two separate functions for malate (or oxalacetate) in fatty acid oxidation; one, to help initiate the oxidation to the stage of acetoacetate (or the hypothetical 2-carbon intermediate); the second, to engage in condensation reactions with the 2-carbon intermediate to form tricarboxylic acid, thereby causing fatty acid oxidation to occur through the Krebs cycle via the 2-carbon unit stage (2).

The requirement of salts or certain non-electrolytes in approximately isotonic concentration for the preparation and activity of the fatty acid oxidase is an unusual finding in the light of known enzyme and protein properties. It is tempting to assume that these particles, catalyzing the reactions of the Krebs cycle and fatty acid oxidation with accompanying aerobic phosphorylations in a highly integrated manner, preexist as such in the intact liver cell. However, examination of isolated nuclei, mitochondria, and microsomes prepared by the method of Schneider (23) for fatty acid oxidase activity has given no positive results, all fractions being completely inactive in the test system described in this paper. This approach is complicated by the strong possibility that the enzyme system becomes inactivated during the course of the fractionation procedure.

SUMMARY

Particulate material separated from rat liver homogenized in isotonic salt solutions, washed with saline, and then suspended in *water* showed no activity in the oxidation of fatty acids when supplemented with ATP, Mg^{++}, and phosphate buffer. When the material was suspended in *saline* instead, the preparations were highly active with the supplements named. However, the fatty acid oxidase activity of the *water* suspension of the particles could be completely restored in the presence of ATP, Mg^{++}, phosphate buffer, cytochrome *c*, neutral salts, or certain non-electrolytes such as sucrose, and catalytic amounts of malate or oxalacetate. The function of the inorganic phosphate was found to lie in its participation in coupled oxidative phosphorylation by the use of P^{32} as a tracer. The action of malate or oxalacetate has two distinct phases: one, to initiate the oxidation of octanoate to the stage of the 2-carbon intermediate, and the second, to cause the latter to enter into the Krebs cycle by forming tricarboxylic acid. The function of the neutral salts in the fatty acid oxidase system appears to lie in the production of an enzymatically active "flocculated" form of the enzyme from an inactive "dispersed" form.

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Addendum—Since this manuscript was prepared for publication, we have published a preliminary report (24) in which data were presented to show that the fatty acid oxidase activity is present exclusively in the mitochondria fraction of rat liver prepared by the method of Hogeboom, Schneider, and Pallade (25).

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RAPID DETERMINATION OF *n*-OCTANOIC ACID*

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The lack of rapid, specific methods for routine determination of the saturated fatty acids has been an impediment in the study of enzymatic reactions of fatty acids. The classical steam distillation and titration technique applied to the higher steam-volatile fatty acids, such as *n*-octanoic acid, in amounts likely to be used in tissue slice or enzyme experiments has been found to be completely inadequate on the basis of the labor and time involved in the distillations, the lack of specificity within the homologous series of steam-volatile fatty acids, and the uncertain accuracy. For instance, Leloir and Muñoz have used the distillation technique in the analysis of octanoic acid in tissue slice media and report consistently incomplete recoveries, with negative errors as large as 27 per cent, in the distillation and titration of 5 to 20 micromole quantities (1).

In this paper is described a method for rapid determination of *n*-octanoate which we have used extensively in this laboratory in the study of the enzymatic oxidation of fatty acids. The method responds only to normal saturated fatty acids having from 7 to 10 carbon atoms, as far as is known, and is analytically useful for *n*-octanoic and *n*-nonanoic acids. Higher and lower fatty acids give no response with this method, nor do any of a large series of compounds likely to be present in biological systems. The method to be described has an effective range of 2 to 7 micromoles of octanoate. In addition, details of a micro modification are given which allow determinations in the range of 0.4 to 1.4 micromole.

Principle of Method—A copper-lime filtrate containing the fatty acid is acidified and extracted with petroleum ether in a glass-stoppered centrifuge tube. The aqueous phase is removed with a capillary pipette. The petroleum ether extract is freed of interfering substances forming insoluble silver salts (chlorides, etc.) by washing with water. The fatty acid is then extracted into 0.1 N NaOH. A sample of the NaOH layer is brought to pH 5.6 with acetic acid and silver nitrate is added, forming a turbidity consisting of insoluble silver octanoate which is stabilized with gum ghatti. The optical density of the turbidity is proportional to octanoate concentration within limits and is measured in a photoelectric colorimeter.

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The extractions and washing are performed in a single vessel, a glass-stoppered, conical tipped centrifuge tube. Since the time factor is not critical in any phase of the determination, the number of samples which may be run simultaneously is limited by the capacity of the shaking apparatus, which is convenient but not essential for the analysis.

The specificity of the method for the saturated normal fatty acids having from 7 to 10 carbon atoms is due to two factors: fatty acids having less than 7 carbon atoms are less readily extracted from water solution by petroleum ether and have relatively soluble silver salts, and fatty acids having more than 10 carbon atoms form insoluble calcium or copper salts and are completely removed in the copper-lime deproteinization treatment.

Reagents—

1. $CuSO_4$ solution. 20.0 gm. of $CuSO_4 \cdot 5H_2O$ per 100 ml. of solution.

2. Calcium hydroxide suspension. 10 per cent suspension in water.

3. Metaphosphoric acid solution. 15.0 gm. made to 100 ml.

4. Skellysolve B (petroleum ether, stated by the manufacturer to be essentially *n*-hexane; boiling range 60–71°).

5. Sodium hydroxide solution adjusted to 0.100 N. This solution must be free of chloride ion.

6. Acetic acid-gum ghatti solution. 13.0 ml. of glacial acetic acid are diluted with approximately 150 ml. of H_2O. To this solution are added 15 ml. of 1 per cent gum ghatti solution. The mixture is made to 500 ml. with H_2O and filtered. The gum ghatti solution is prepared by suspending 1.0 gm. of gum ghatti (Sargent) in a gauze bag in 100 ml. of H_2O overnight at room temperature, followed by filtration. The combined reagent is stable for as long as 6 weeks at room temperature. This solution must be chloride-free.

7. 1.5 M silver nitrate.

8. Stock standard octanoate solution. 1.58 ml. of *n*-octanoic acid (Eastman, redistilled) are dissolved in 50 ml. of warm 0.21 N NaOH with shaking and made to 100 ml. with H_2O. This solution, which is 0.10 M (100 micromoles per ml.) is kept in the refrigerator. Before use it is allowed to warm to 30° and shaken thoroughly.

Apparatus—

1. 40 ml. glass-stoppered, conical tip centrifuge tubes. Maizel-Gerson reaction vessels (without stop-cock, supplied by the Wilkens-Anderson Company, 111 North Canal Street, Chicago) have been used in this laboratory.

2. Shaking machine. We have used the International No. 2 machine, adapted to hold sixteen tubes. It is recommended that the shaking times necessary for complete extraction of fatty acid into and out of Skellysolve be determined for the particular machine and shaking rate used.

3. Capillary pipettes, drawn from glass tubing. These are connected with rubber tubing to a ground glass stop-cock, which in turn is connected with flexible rubber tubing to a large suction flask and a water aspirator.
4. 4.0 ml. transfer pipettes.
5. Tubes to fit the photoelectric colorimeter. The method as described was designed to yield 5.2 ml. of suspension to be read in the Evelyn photoelectric colorimeter, with the 6 ml. opening with standard Evelyn tubes.

Procedure

Extraction of Octanoate into Skellysolve B—An aliquot of protein-free filtrate (the deproteinization procedure is considered in a separate section) containing between 2 and 7 micromoles of octanoate (0.20 to 0.70 ml. of 0.01 N acid) is placed in a 40 ml. glass-stoppered, conical tip centrifuge tube and 1.0 ml. of 15 per cent metaphosphoric acid is added. The volume is made to 10.0 ml. with H_2O. To the mixture are added 10.0 ml. of Skellysolve B. The tubes are stoppered and shaken for 5 minutes. The aqueous phase (lower layer) is then removed from each tube by means of the drawn out capillary pipettes attached via rubber tubing and a glass stop-cock to a water aspirator. Removal of the aqueous layer can be made essentially complete, since the conical tip of the tube and the control provided by the glass stop-cock allow removal of all but a very small drop of the aqueous phase.

Washing of Skellysolve Extract—To each tube are added 10.0 ml. of H_2O (chloride-free), the water being allowed to rinse the surface of the ground glass joint. The tubes are restoppered (the stoppers meanwhile having been rinsed in a stream of chloride-free water) and again shaken for 5 minutes. The aqueous layer is removed as completely as possible, again with capillary pipettes and a suction line.

Extraction of Octanoate into Aqueous NaOH—To each tube are added 5.0 ml. of 0.100 N NaOH. The tubes are stoppered and again shaken for 5 minutes.

Development of Turbidity—A 4.0 ml. sample of the NaOH layer is removed with a 4.0 ml. transfer pipette. The upper end of the pipette is closed with the pipette finger as the pipette is passed down through the upper layer of Skellysolve to prevent removing any solvent with the aqueous layer. The pipette is wiped with gauze to remove adhering Skellysolve and the contents delivered into a colorimeter tube containing 1.0 ml. of the acetic acid-gum ghatti solution. The contents are mixed by shaking. To each tube is added 0.20 ml. of 1.5 M $AgNO_3$, causing the formation of a turbidity due to silver octanoate. The contents are immediately mixed by shaking. The turbidities are read in a photoelectric colorimeter with a 660 mμ filter. The tubes are shaken just before the reading is taken.

Ordinarily we have made the readings 15 minutes after addition of the silver nitrate, but the turbidities remain constant for an hour.

Colorimeter Blank—The blank tube contains 4.0 ml. of 0.1 N NaOH, 1.0 ml. of the acetic acid-gum ghatti solution, and 0.20 ml. of 1.5 M $AgNO_3$. This tube is used to set the galvanometer (in the case of the Evelyn instrument) at 100.

Standard Curve—A standard curve relating micromoles of octanoate to colorimeter readings is prepared for each new batch of reagents. The standard curve obtained can be consistently duplicated with the same batch of reagents; therefore in practice only a single standard is run with each set of determinations. The stock standard solution (100 micromoles per ml.) is diluted 1:100 to prepare a working standard containing 1.0 micromole of fatty acid per ml. Aliquots corresponding to between 1.0 and 8.0 micromoles are carried through the entire procedure, including the deproteinization steps. In Fig. 1 is shown a typical standard curve prepared for octanoic acid. Only the linear portion of the curve is used, since below 2.0 and above 7 to 8 micromoles of fatty acid the turbidity is no longer linear.

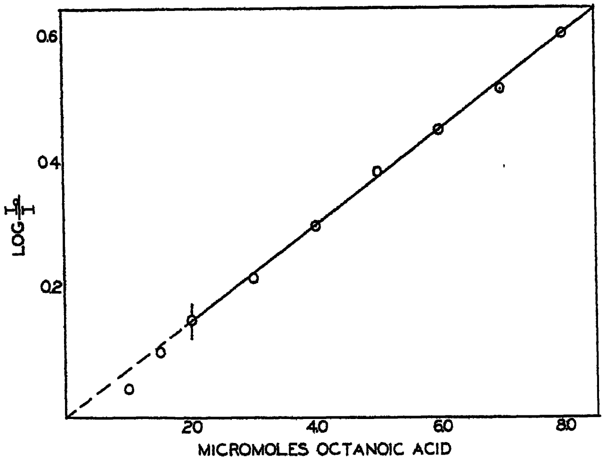

FIG. 1. Standard curve for determination of n-octanoic acid

Average Error of Single Determinations—Several sets of experiments to test the reliability of single determinations and actual use of the method applied to studies of the fatty acid oxidase system (see the preceding paper) indicate that the average error of a single determination does not exceed 3.5 per cent. This figure compares very favorably with the values obtained by Leloir and Muñoz (1) for distillation and titration of octanoic acid in amounts ranging from 5 to 20 micromoles. They reported consistently incomplete recoveries, with errors ranging from −16 to −27 per cent.

Deproteinization—A study was made of various deproteinization procedures to determine the best method from the standpoint of completeness of recovery and elimination of interfering materials which cause formation of emulsions. The modified copper-lime treatment described below is the only one of many procedures tested which proved to be satisfactory. Trichloroacetic acid, tungstic acid, metaphosphoric acid, and heat coagulation were found to cause *n*-octanoic acid to become adsorbed on the coagulated protein to such an extent that in many cases no octanoic acid whatsoever could be recovered in the filtrate. This is not particularly surprising, since the fatty acids, having 7 or more carbon atoms, are very sparingly soluble in acid solution and are highly surface-active. However, the alkaline conditions prevailing in the copper-lime treatment favor complete recovery of heptanoic, octanoic, and nonanoic acids from protein-containing mixtures. Even with this method of deproteinization there are definite limitations to the conditions under which fatty acid may be recovered completely from protein-containing mixtures. For this reason conditions must first be established for complete recovery of fatty acid from the material to be analyzed.

The method has been used for the determination of fatty acid added as substrate to a buffered medium in which tissue slices were shaken in Warburg vessels. In such experiments no difficulty was experienced in recovering added octanoate. After completioe of the experiment the slices were removed from the Warburg vessels and an aliquot of the medium removed for treatment as described below.

Fatty acid determinations in tissue suspensions or homogenates, however, are subject to definite limitations because the ratio of fatty acid concentration to tissue concentration is critical in obtaining complete recovery of fatty acid by the deproteinization procedure described below or by variations thereof. In Table I are shown recoveries of a standard amount of octanoate from a standard volume of mixture containing variable amounts of rat liver homogenized in water. It can be seen that as the tissue concentration is increased a point is reached at which the recovery of a known amount of octanoate falls off considerably.

In general, octanoate may be recovered quantitatively from suspensions containing as much as 10 per cent tissue, provided the suspension is diluted with at least an equal volume of water before application of the copper-lime treatment. The concentrations of copper sulfate an cdalcium hydroxide suspension used in the method as described below are optimal; increasing these concentrations or altering their ratios does not lead to increased recovery at very high tissue concentrations.

In Table II are shown recoveries obtained in a case in which conditions were satisfactory for complete recovery of a wide range of octanoate concentrations. The experiments described in Tables I and II may be used as a guide in establishing the proper deproteinization method when analysis of fatty acid in given enzyme preparations or bacterial suspensions is desired.

TABLE I

Effect of Tissue Concentration on Recovery of Standard Amounts of n-Octanoic Acid from Rat Liver Homogenate

Each tube contained 1.00 ml. of octanoate (12.0 micromoles), water, and rat liver homogenate to make a total volume of 6.0 ml. Immediately after mixing, 6.5 ml. of H_2O were added, followed by 1.0 ml. of $CuSO_4$ solution and 1.50 ml. of $Ca(OH)_2$ suspension. Octanoic acid recovery was determined in aliquots of the filtrate. The liver homogenate contained 250 mg. of fresh tissue per ml.

Volume of homogenate added	Octanoic acid recovered	Volume of homogenate added	Octanoic acid recovered
ml.	*micromoles*	*ml.*	*micromoles*
0.0	12.1	2.5	10.9
0.5	12.0	3.0	10.2
1.0	12.0	4.0	4.1
1.5	11.8	5.0	2.0
2.0	12.2		

TABLE II

Recovery of Octanoate from Rat Liver Homogenate under Favorable Conditions

Each tube contained 4.0 ml. of octanoate (0 to 24.0 micromoles, as indicated) and 2.0 ml. of rat liver homogenate (250 mg. of tissue per ml.). Immediately after mixing, 6.5 ml. of H_2O were added, followed by 1.0 ml. of $CuSO_4$ and 1.50 ml. of $Ca(OH)_2$ suspension. Octanoic acid was determined in duplicate on 5.0 ml. aliquots of filtrate.

Octanoic acid added	Octanoic acid recovered	Octanoic acid added	Octanoic acid recovered
micromoles	*micromoles*	*micromoles*	*micromoles*
24.0	24.4	10.0	10.2
	24.5		9.8
18.0	17.9	9.0	9.0
	17.6		8.8
15.0	15.3	6.0	5.8
	14.8		5.8
12.0	12.0	0.0	0.0
	11.8		0.0

The deproteinization treatment found best for analysis of fatty acid in tissue slice media, tissue homogenates, and washed suspensions of ground tissues (2) follows: The sample, containing between 6.0 and 21 micromoles of octanoate, is diluted with water to 12.5 ml. 1 ml. of the $CuSO_4$ solution is added and mixed, and this is followed by the addition of 1.5 ml. of $Ca(OH)_2$ suspension. The contents are thoroughly mixed and allowed to stand 15 minutes. The tubes are centrifuged and the supernatants filtered through paper. The volume of filtrate is enough for analysis of two aliquots of 5.0 ml., containing from 2.0 to 7.0 micromoles of fatty acid, which are carried through the procedure as already outlined.

Metaphosphoric acid is used as the acidifying reagent in the extraction procedure because it serves to prevent formation of emulsions due to traces of protein which escape removal in the copper-lime treatment.

TABLE III

Specificity in Homologous Series of Saturated Normal Acids

The indicated amounts of the following fatty acids were carried through the entire analytical procedure including the copper-lime treatment, and the turbidities resulting were expressed in terms of the micromoles of octanoate giving equal turbidity readings.

Fatty acid; No. of C atoms in chain	Amount in sample	Octanoic acid equivalent
	micromoles	*micromoles*
2	1000	No turbidity
4	300	" "
5	500	0.9
6	50	0.6
7	15	3.4
	10	No turbidity
8	5	5.0
9	5	3.7
10	5	2.1
12	200	No turbidity
14	200	" "
16	200	" "

Specificity—The following observations indicate the specificity of the method. All normal saturated fatty acids up to and including *n*-nonanoic acid may be completely recovered in the copper-lime treatment outlined above. *n*-Decanoic acid cannot be completely recovered; when 10 micromoles of *n*-decanoate were subjected to the copper-lime procedure outlined, only 4.5 micromoles could be recovered. Fatty acids having 12 or more carbon atoms are *completely* removed by the copper-lime procedure, even when relatively large amounts are present owing to the insolubility of their copper or calcium salts. Of the fatty acids having fewer than 10 carbon atoms only octanoic and nonanoic acids can be determined by the method outlined, since only these acids show a linear relationship between optical density and concentration. Although 15 micromoles of heptanoic acid give a response equal to that given by 3.4 micromoles of octanoate, there is no linearity of response. Fatty acids having less than 7 carbon atoms must be present in relatively enormous concentration to yield even a slight turbidity. The data presented in Table III indicate the magnitude of turbidity responses given by different fatty acids.

In order to test the specificity of the method with respect to compounds other than saturated fatty acids, mixtures containing 1.0 ml. of 15 per cent metaphosphoric acid, 5.0 ml. of octanoate (5 micromoles) or 5.0 ml. of H_2O, and 4.0 ml. of a solution containing 150 micromoles of the substance to be tested for interference were carried through the entire procedure (the deproteinization step was omitted). The following compounds gave absolutely no turbidity under these conditions nor did they interfere with the recovery of octanoate: sodium chloride, sodium sulfate, magnesium sulfate, monosodium phosphate, sodium borate, sodium fluoride, potassium iodoacetate, ammonium sulfate, sodium arsenate, trichloroacetic acid, tungstic acid, glucose, fructose, xylose, glycogen, glycine, cysteine, acetylcholine, *p*-aminobenzoic acid, aniline acetate, α-aminooctanoic acid, ascorbic acid, alloxan, sodium benzoate, glycolic acid, glycerol, creatine, ethyl alcohol, acetaldehyde, lactate, pyruvate, acetopyruvate, citrate, α-ketoglutarate, succinate, fumarate, *l*-malate, oxalacetate, *cis*-aconitate, acetoacetate, acetate, malonate, β-glycerophosphate, fructose diphosphate, sodium pyrophosphate, adenosine triphosphate, β-hydroxybutyrate, β-hydroxyoctanoate, crotonate, sorbate, Δ^1- and Δ^2-hexenoate, α,γ-diketooctanoate, acetoin, *l*-glutamate, and glutamine.

The responses given by unsaturated acids of intermediate chain length, branched chain acids, or halogenated acids have not been more extensively examined. If such compounds are suspected to be present in the material to be analyzed, the specificity of the response must of course be examined further.

Efficiency of Extractions—The extractions of octanoate from the aqueous phase and from Skellysolve back into aqueous NaOH are essentially complete. This was determined by preparing a series of turbidities developed from octanoate in 0.1 N NaOH directly, without going through the extraction procedure, and comparing the responses given with those obtained when the same amounts of octanoate were carried through the whole procedure, allowance being made for the aliquot of NaOH extract used for turbidity development. The standard curves obtained in the two cases are identical. This indicates that the extractions are complete and that washing the extract with water does not cause any measurable loss of fatty acid.

Effect of Variations in Volume of Aqueous Phase Extracted—Although the volume of the aqueous phase extracted has been specified as 10.0 ml., it may be varied between 5.0 and 20.0 ml. with essentially complete recoveries. Use of a large aqueous phase may be necessary for analysis of filtrates in which the fatty acid concentration is very low.

Efficiency of Washing Procedure—The conditions of analysis suggest that a possible point of difficulty might lie in the washing of the Skellysolve extract to free it of chlorides or other substances which form insoluble silver salts. When such substances are present in the concentrations likely to be found in biological fluids or in enzyme reaction media, absolutely no interference has been encountered. When 9.0 ml. of saturated NaCl solution acidified with 1 ml. of metaphosphoric acid were carried through the analysis, only a faint turbidity (due to AgCl), corresponding to less than 0.4 micromole of octanoate, was observed. The failure of chlorides in ordinary concentrations to produce turbidities indicates that the washing of the Skellysolve extract containing the fatty acid is extremely efficient in removing traces of the original aqueous phase from the centrifuge tubes used in the analyses. We have never experienced any difficulties due to contamination of reagents or glassware with chlorides or other substances capable of reacting with silver ions.

Conditions Affecting Turbidity Response—The pH of the acetate-buffered solution in which the turbidity is developed is 5.6. It has been found that the medium must be well buffered to obtain reproducible turbidities. Raising the pH substantially causes silver acetate to be coprecipitated and the optical densities of the turbidities given by standard amounts of octanoate are no longer linear. Lowering the pH causes, as would be expected, a great decrease in the sensitivity, since the silver octanoate becomes more soluble. The concentration of acetate chosen is high enough to give maximal stability of response without causing any interference by precipitation of silver acetate.

The omission of gum ghatti from the turbidity mixture greatly reduces the stability of the turbidity and also reduces the useful range in which optical density is linear with octanoate concentration, since at the extremes the silver octanoate flocculates out immediately or after short standing. Gum arabic, agar, soluble starch, and gelatin have also been tested but gum ghatti is the most effective agent for stabilizing the turbidities. When the concentration of gum ghatti is greatly increased, the range of response is decreased, since the lower concentrations of octanoate then fail to produce a turbidity with silver ions.

The concentration of silver ions is also critical in the optical density produced, although 10 per cent errors in the addition of the silver nitrate solution cause no significant change in optical density. Substantial decreases in concentration of silver ion cause concomitant decreases in sensitivity, since the solubility product of silver octanoate is not exceeded with the lower range of octanoate concentrations. Increasing the concentration of silver ion brings about an increasing tendency for silver acetate to precipitate after short standing.

Attempts to combine the silver nitrate and the acetic acid-gum ghatti reagents for greater convenience in analysis met with failure, since addition of such a combined reagent to the 0.1 N NaOH solution of octanoate causes precipitation of silver oxide which does not immediately redissolve on mixing, owing probably to the presence of the gum ghatti. The turbidities developed under these conditions have been found to be very erratic.

The turbidities of silver octanoate are not photosensitive over a period of several hours and no special precautions have been found necessary to control this factor.

Micro Modification—Although the method as outlined above has been found to be quite suitable in routine analysis of large numbers of samples, in certain cases the small size of the samples available did not permit use of the method in the useful range. For contingencies of this type we have devised a modification in which the working procedure is identical but in which volumes are reduced in scale by a factor of 5. Samples containing 0.4 to 1.4 micromole of octanoate are acidified with 0.2 ml. of HPO_3 and made to 2.0 ml. They are extracted with 2.0 ml. of petroleum ether in a 10 ml. conical tipped tube, the extracts washed with 2.0 ml. of H_2O, and the octanoate brought into 1.0 ml. of 0.1 N NaOH. An 0.8 ml. sample of the NaOH layer is added to 0.2 ml. of the acetic acid-gum ghatti and the resulting mixture is treated with 0.04 ml. of 1.5 M $AgNO_3$. The turbidities are read at 660 $m\mu$ in a Beckman quartz spectrophotometer equipped with a diaphragm adapter to produce a "pinhole" light source (3). The position of the beam was adjusted so that it passed through a standard 3.0 ml. Beckman cell mounted on an adjustable block in the cell holder without striking the bottom or walls of the cell or the meniscus when the cells contained 1.0 ml. samples. The general principles outlined by Lowry and Bessey (3) for microspectrophotometric measurements were observed. The errors were not significantly greater than in the macro method.

SUMMARY

A simple method for the rapid determination of octanoic acid in small amounts has been described. The method involves deproteinization with mixtures of copper sulfate and calcium hydroxide, acidification of the filtrate, and extraction of the fatty acid into petroleum ether. The extract is washed with H_2O and the fatty acid then extracted into 0.1 N NaOH. An aliquot of the aqueous phase is brought to pH 5.6 by addition of acetic acid. The addition of silver ions produces a turbidity of silver octanoate, stabilized with gum ghatti, the optical density of which is proportional to oc-

tanoic acid concentration. The method responds only to *n*-octanoic acid and immediately adjacent homologues. A large variety of compounds likely to be present in enzyme reaction media were found not to interfere with the determination.

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PREPARATION OF RADIOACTIVE CARBON-LABELED SUGARS BY PHOTOSYNTHESIS*

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Carbohydrates, glucose, fructose, sucrose, and starch, labeled in all carbon positions with C^{14}, are useful for the study of intermediate metabolism in living organisms and for many other purposes. Two papers dealing with the synthesis of labeled carbohydrates have already appeared (1, 2), but no detailed methods of preparing the above carbohydrates are available. It is the purpose of this paper to describe such methods.

Radioactive starch, glucose, fructose, and sucrose can be isolated from green leaves in good yields after they have been exposed to an atmosphere of radioactive carbon dioxide in the presence of light. At the end of the photosynthetic period, the leaf or leaves are extracted with dilute alcohol. The alcohol-insoluble fraction contains the starch which can be isolated. Hydrolysis of the starch with acid produces glucose which can be obtained in crystalline form. The alcohol extract contains glucose, fructose, and sucrose. In order to increase the yield of the monosaccharides, the sucrose is hydrolyzed with acid, thus producing additional glucose and fructose. The separation of fructose from glucose is accomplished by precipitating the former with calcium hydroxide. The calcium complex is then decomposed and the fructose liberated. Sucrose is separated from the alcoholic extract. The two monosaccharides are first fermented with *Torula monosa* and then the residual sucrose is concentrated and crystallized.

Apparatus—The photosynthetic chamber is made of a Pyrex glass tube (Fig. 1) 21 cm. in length having an inside diameter of 5.5 cm. The lower end consists of a drawn down, recurved entry tube fitted with a stop-cock, *a*, and a 10/30 tapered joint. The upper end bears a 60/50 joint, A_1, to which a cover is fitted, carrying an exit tube which is also fitted with a stop-cock, *b*, and a 10/30 joint. The volume between stop-cocks *a* and *b* is 500 ml.

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When conducting an experiment, about 500 mg. of barium carbonate containing approximately 0.25 millicurie of C^{14} are introduced into a 25 ml. Erlenmeyer flask, *B*, from which the neck has been removed. A cork disk, *e*, slightly smaller than the inside diameter of chamber *A*, is fitted to the top of the flask so that its position is fixed when placed in the chamber. The barium carbonate is then mixed with about 5 ml. of water and a few drops of paraffin oil are added to prevent excessive foaming. 1 ml. of 80 per cent lactic acid is placed in a small test-tube, *f*, cut off so that it rests at about a 45° angle when placed in flask *B*. This flask, containing the barium carbonate slurry and the test-tube of lactic acid, is then placed in an upright position in the photosynthetic chamber, *A*, which was previously rinsed with water to assure a humid atmosphere. Enough water should also be left in the recurving portion of the entry tube to indicate when the internal and external pressures are equalized.

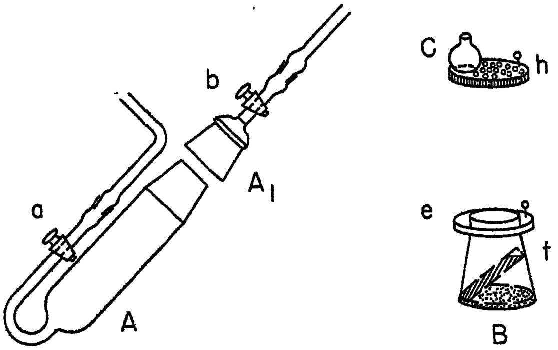

FIG. 1. Photosynthetic chamber, Erlenmeyer flask, and Florence flask

A 10 ml. Florence flask, *C*, from which the neck has been removed is attached to a plywood disk, *h*, bored through with numerous holes. The flask is filled with water and the petiole of the leaf, which had previously been kept in the dark for 24 hours, is inserted so that it reaches the bottom of the flask. The leaf and its container are placed in a vacuum desiccator and evacuated to about 20 cm. After the initial flow of gas bubbles from the petiole of the leaf ceases, the pressure is equalized and the water displaced in flask *C* is replaced. The leaf and container are then placed in the photosynthetic chamber, *A*, on top of the cork ring, *e*. A piece of moistened filter paper is placed over the plywood disk to prevent spattering of acid into the upper part of the vessel. The greased, upper end, ground taper, A_1, is fitted on, and with the entry tube, *a*, closed, the chamber is partially evacuated through the exit tube which is then closed with stop-cock *b*. By tilting the chamber about 30° the acid is dumped into the barium carbonate slurry. This should be done carefully to avoid excessive foaming. When the reaction has subsided and all the barium carbonate has reacted, liberating the carbon dioxide, atmospheric pressure is restored by opening stop-cock *a*. The chamber is then immersed in a cylindrical Pyrex water bath, 10 inches high and 18 inches in diameter, held in position by a condenser clamp on a heavy ring-stand in the bath as shown in Fig. 2.

Illumination is effected by two 100 watt bulbs in desk lamps, g_1 and g_2, placed opposite each other on the outside of the bath. A small fan (not shown in Fig. 2) is placed above the water bath so that a current of air passes over the surface of the bath. This maintains the bath temperature 3–4° above the prevailing room temperature. Illumination is continued for 18 to 24 hours. A relatively long period of illumination is used in order to nsure complete utilization of the available carbon dioxide and to produce imore uniformly labeled compounds.

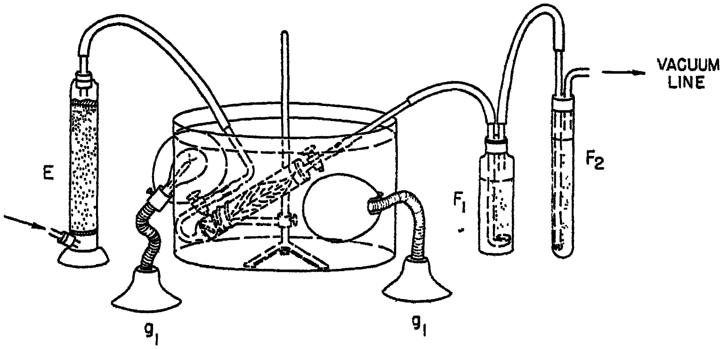

FIG. 2. Complete apparatus for photosynthesis

During the last hour of illumination a soda lime tower, *E*, is attached to the entry tube of the photosynthetic chamber and two carbon dioxide traps, F_1 and F_2, are attached in series to the exit tube. During this period about 5 liters of carbon dioxide-free air are pulled through the system by applying a vacuum at F_2. Trap F_1 consists of a 500 ml. jar fitted with a sintered glass aerator containing 335 ml. of 0.1 N sodium hydroxide; trap F_2 consists of a 100 ml. test-tube also equipped with an aerator and 65 ml. of 0.1 N sodium hydroxide.

Usually no appreciable amount of carbon dioxide is found in traps F_1 and F_2 at the end of the illumination period.

Plant Materials—Turkish tobacco leaves proved to be good starch producers. A high rate of synthesis can be achieved after the leaves are placed in the dark for approximately 24 hours to use up the reserve carbohydrate and are then subjected to photosynthesis in an atmosphere initially containing 10 to 15 per cent carbon dioxide. It has been observed that under the experimental conditions described about 20 per cent of the dry weight of the alcohol-extracted leaf residue consists of starch.

The plants are grown in Hoagland's solution or sand culture. The leaves, weighing from 3.0 to 3.5 gm. and being from 15 to 18 cm. in length, are taken from the middle portion of the stem of plants about 2.5 feet high. Leaves of plants grown in culture solution are harvested in the morning and placed in water in the dark until the following morning. Plants grown in pots of sand are placed with the container in the dark for a similar period of time and the leaves harvested just prior to the experiment. On a fresh weight basis the starved leaves usually contain about 0.25 per cent reducing sugars (glucose and fructose) and 0.15 per cent sucrose. The residue does not give a blue color with iodine, indicating the absence of starch. After 24 hours of illumination in the chamber in an atmosphere of carbon dioxide derived from 0.5 gm. of barium carbonate, about 0.85 per cent reducing sugars, 0.65 per cent sucrose, and 2.5 per cent starch are found.

Labeled Starch—Several lots of radioactive starch were prepared by the following method: A Turkish tobacco leaf was harvested in the morning and allowed to remain in the dark for 24 hours to exhaust the starch. The leaf was then placed in the photosynthetic chamber in an atmosphere of radioactive carbon dioxide, as previously described, and illuminated for 24 hours. At the conclusion of the photosynthetic period and after the chamber had been swept out with carbon dioxide-free air, the leaf was killed by immersion in boiling 80 per cent alcohol. It was then cut into small pieces and placed in a Soxhlet extraction thimble. The alcohol used in killing the leaf was transferred to a boiling flask of a Soxhlet extractor. The extractor was assembled and the extraction continued for 6 to 8 hours, after which the alcoholic extract containing the soluble sugars (glucose, fructose, and sucrose) was set aside.

After the 80 per cent alcohol extraction, the residue remaining in the Soxhlet thimble was dried at 50° in a vacuum oven for a period of 18 to 24 hours (0.31 to 0.42 gm. of dry material was obtained), 25 per cent of its weight of magnesium carbonate was added, and the mixture finely ground in a mortar under a hood. The ground material was extracted by the method of Pucher and Vickery (3) as follows: It was transferred to a heavy duty 50 ml. centrifuge tube to which an amount of 100 to 150 mesh ground glass, equivalent to 7.5 times the weight of the plant material, was added. This was followed by the addition of 5 ml. of water and a heavy stirring rod was placed in the tube. The tube with the contents was immersed in a steam bath and vigorously stirred[1] for 15 minutes to gelatinize the starch. 7 ml. of 46 per cent calcium chloride, previously heated on the same water bath, were added and stirring was continued for 10 minutes. The tube was centrifuged, the supernatant liquid decanted into a 100 ml. centrifuge tube, and the residue ground again for about 5 minutes. The tube was returned to the steam bath, 3 ml. of boiling water and 5 ml. of 46 per cent calcium chloride at 100° were added, and stirring continued for 10 minutes. The tube was centrifuged and the supernatant liquid decanted into the 100 ml. tube containing the first extract. After four such extractions, the last drop of the fourth supernatant liquid was acidified with a drop of 0.5 N acetic acid and tested with dilute iodine solution. If no blue starch-iodine color was observed, the extraction of starch was assumed to be complete and the residue was washed twice with 15 ml. of hot water. If the starch-iodine test was positive, the extraction procedure was continued until a negative test was obtained. Seven or eight extractions are sometimes necessary to free the residue of starch completely.

The extract (60 to 120 ml.) in the 100 ml. centrifuge tube (or tubes) was treated with 1 ml. of 2.3 N hydrochloric acid and 2 ml. of 20 per cent sodium chloride per each 10 ml. of extract. The starch was then precipitated by the addition of 0.5 ml. of iodine-iodide solution (30 gm. of iodine and 50 gm. of potassium iodide diluted to 250 ml.) per 10 ml. of extract. The tube was loosely stoppered and allowed to stand for 10 minutes, after which it was placed in a steam bath for 15 minutes. The tube and contents were cooled, centrifuged, the supernatant liquid decanted, and the starch-iodine complex well washed with 60 per cent alcohol. The complex was then decomposed with 2 to 4 ml. of 0.25 N alcoholic sodium hydroxide and washed three times with 60 per cent alcohol. The crude starch was dissolved in 5 ml. of water, filtered through a filter paper into a centrifuge tube, and reprecipitated with 1½ volumes of 95 per cent alcohol. When precipitation was complete, the starch was centrifuged, washed with 60 per cent alcohol,[2] twice with 95 per cent alcohol, twice with absolute alcohol, and finally twice with ether. After the ether had evaporated and the starch was ground to a powder, it was placed in a vacuum oven and dried at 50° for 24 hours.

Several starch preparations made by this method with approximately

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[1] The extraction of the starch from the plant material can also be effected by homogenization as described by Umbreit, Burris, and Stauffer (4).

[2] If the starch is to be used immediately for preparing radioactive glucose, the drying is unnecessary; it can be immediately hydrolyzed with sulfuric acid after washing with 60 per cent alcohol, as described in "Preparation of labeled glucose from starch."

230 microcuries of C^{14} in 0.5 gm. of barium carbonate yielded from 20 to 25 per cent starch calculated on a dry basis of the alcohol-extracted plant material. The specific activity of the starch was from 0.48 to 0.72 microcurie per mg.

Radioactivity was measured by spreading a weighed amount of the starch on an aluminum plate with the aid of alcohol and, after drying at 50° *in vacuo* for 24 hours, assaying the sample with a bell jar type counter, as described by Kamen (5).

Preparation of Labeled Glucose from Starch—The radioactive starch was hydrolyzed to glucose by dissolving it in sufficient water to make a 0.2 per cent solution, at the same time adding an amount of inactive glucose carrier necessary to provide enough material to crystallize conveniently (0.3 gm. was usually added). An equal volume of 2 N sulfuric acid was added to this solution and the mixture refluxed for 30 minutes on a hot-plate. After cooling, the theoretical amount of powdered barium carbonate needed to neutralize the acid was added and the precipitate centrifuged off and washed. The supernatant liquid and the washings were then passed through Duolite ion exchange columns, C-3 and A-3, having 25 ml. bed volumes. Each column was washed with 100 ml. of water. The demineralized solution, about 300 ml., was concentrated to a small volume *in vacuo* at 50°, transferred to a 25 ml. beaker, and taken to a thick sirup in the vacuum oven at 50°. The sirup was warmed on the steam bath and approximately 4 volumes of hot absolute alcohol were stirred in. To the resultant viscous mass 50 mg. of finely powdered crystalline glucose were stirred in and the beaker was allowed to cool in a desiccator. Crystallization of the glucose was complete within 24 hours. The crystals were transferred to a sintered glass funnel with cold absolute alcohol, sucked dry, washed with ether, and placed in the vacuum oven at 50°. After 24 hours the sugar was ground in a mortar, weighed, and assayed for radioactivity.

An 85 mg. starch sample, having a specific activity of 0.57 microcurie per mg. and a total activity of 48.6 ± 5.9 microcuries, yielded, after addition of 350 mg. of inactive glucose, 406 mg. of glucose with a specific activity of 0.09 microcurie per mg. and a total activity of 36.7 ± 0.63 microcuries.

Another 71 mg. starch sample with a specific activity of 0.48 microcurie per mg. yielded, after addition of 350 mg. of inactive glucose, 383 mg. of glucose having a specific activity of 0.1 microcurie per mg.

Preparation of Labeled Fructose—The 80 per cent alcohol plant extract contains glucose, fructose, and sucrose. Most of the labeled glucose can be crystallized out by adding inactive glucose to the mixture after acid hydrolysis of the sucrose. The fructose, which does not readily crystallize and remains in solution, can be separated from glucose by forming the insoluble calcium-fructose complex, which is later decomposed.

The technique used in the preparation of fructose was as follows: The 80 per cent alcoholic extract was concentrated on the steam bath with occasional additions of water until all the alcohol was removed. The aqueous solution was cooled and twice extracted, with a separatory funnel, with one-fourth of its volume of ether to remove pigments and tarry matter. The ether was then washed with water and the washings added to the original aqueous phase. This latter solution was returned to the steam bath and heated to remove the dissolved ether. About 400 mg. of inactive glucose carrier were added, and, after the solution was cooled, it was passed through Duolite ion exchange columns, C-3 and A-3, of 25 ml. bed volume capacity, to remove the organic acids and amino acids. Approximately 100 ml. of wash water was used for each column, and the resulting neutral solution concentrated under reduced pressure at 50° to a volume of 8 ml. 2 ml. of 5 N sulfuric acid were added and the solution heated in a water bath at 80° for 10 minutes to invert the sucrose. After cooling and diluting to about 50 ml., the solution was passed through the anion exchange column, A-3, to remove the acid used for hydrolysis. The resulting neutral solution of glucose and invert sugar was concentrated under a vacuum at 50° to a volume of 10 ml., transferred to a small beaker, and concentrated to a thick sirup in the vacuum oven. The glucose was then crystallized as previously described. When the mother liquor was concentrated, a second crop of crystalline glucose was obtained. The specific activity of this glucose was about 25 per cent greater than that of the first crop, indicating radioactive contamination. 200 mg. of inactive glucose were added to the mother liquor in order to reduce the activity of the residual glucose. Upon crystallization, 221 mg. of glucose were recovered with a specific activity equivalent to one-third of that of the second crop. Considering the dilution with inactive glucose, approximately one-tenth of the previous activity would be expected. The higher activity indicates that some radioactive fructose was carried down with the inactive glucose (see Table I).

TABLE I

Results of Crystallizations of Glucose Solution

Crop of glucose	Weight	Specific activity	Total activity
	mg.	*microcurie per mg.*	*microcuries*
1st	426	0.089	38
2nd	79	0.113	9
3rd	221 (Includes 200 mg. carrier)	0.037	8.1

The radioactive glucose obtained from the mixture with fructose was contaminated with about 1 to 2 per cent radioactive fructose. It could be freed almost completely from the radioactive contaminant by adding inactive fructose and recrystallizing.

The calcium-fructose complex was prepared according to Bates and his associates (6). 1 gm. of fructose carrier was added to the mother liquor after practically all of the radioactive glucose had been crystallized out by the addition of inactive glucose and the alcohol present removed by evaporation on the steam bath. In order to precipitate this quantity of fructose, an amount of calcium oxide equivalent to half of the weight of the fructose should be added. A 19 per cent lime suspension was made up from 0.76 gm. of calcium hydroxide powder and 3.25 ml. of water, making a total weight of about 4 gm. The calcium hydroxide used was shown to contain 66 per cent calcium oxide, as determined by titration with 0.1 N hydrochloric acid. The sugar solution containing the radioactive fructose was diluted with 11.7 ml. of water so that the fructose would constitute six per cent by weight of the total reaction mixture.

The fructose solution and the calcium oxide slurry were placed in a refrigerator at 0°. When the fructose solution was cooled to 0°, one-fourth of the lime slurry was poured into a 50 ml. centrifuge tube in an ice bath and then one-fourth of the sugar solution was slowly added with vigorous mechanical stirring. After 15 minutes fine crystalline needles of calcium-fructose could be observed under the microscope. The second quarter of the slurry was added with continuous stirring, followed by the slow addition of the second quarter of the sugar solution. The third and fourth portions were added at 20 minute intervals and after an additional 20 minutes stirring the mixture was placed in a refrigerator overnight. The next day the precipitate was centrifuged in a chilled centrifuge cup and washed twice with 2 ml. portions of ice-cold saturated calcium hydroxide.

The calcium-fructose complex was then treated with an excess of 1 M oxalic acid, with phenolphthalein as an indicator, and stirred with a mechanical stirrer. The calcium oxalate was centrifuged off and washed twice with 25 ml. portions of water. The supernatant liquid and washings were then passed over ion exchange columns, Duolite C-3 and A-3, concentrated in a vacuum below 50° to a small volume, and finally taken to a sirup in a vacuum oven.

This sirup, containing practically all fructose, was crystallized according to Fischer and Baer (7). It was taken up in a small amount of warm absolute alcohol, a few ml. of dry benzene were added, and the mixture was taken to dryness under reduced pressure, while a stream of dry air blew over the surface of the liquid. After repeating this procedure four times, the residue was taken up in a minimum quantity of hot absolute methyl alcohol, cooled in a desiccator, seeded with 50 mg. of finely powdered crystalline fructose, and the fructose precipitated by the slow dropwise addition of absolute ether. The crystals were allowed to remain in a desiccator for 24 hours, transferred to a sintered glass crucible with a mixture of equal volumes of methanol and ether, filtered off, and washed with ether. A yield of 0.925 gm. of fructose was recovered having a specific activity of 0.0325 microcurie per mg. (Table II).

Initially there was a total of 132 microcuries in the sugar solution to which 2.125 gm. of inactive glucose and fructose were added. The radioactive sugars synthesized in the plant probably amounted to about 100 mg. These preparations were made from the combined 80 per cent alcoholic extracts of two tobacco leaves.

The extent to which glucose and fructose were contaminated with one another was found from a control experiment performed as follows: A tobacco leaf, which was previously placed in the dark and then illuminated in the presence of inactive carbon dioxide for 24 hours, was extracted with alcohol, the alcoholic extract clarified, and the sucrose hydrolyzed. The solution containing glucose and fructose was divided into two halves. To one-half of the solution, labeled fructose was added and the glucose crystallized as previously described. The other half was treated with labeled glucose and the fructose was isolated from the mixture by the calcium precipitation method.

TABLE II

Recovery from Preparations of Tobacco Leaves

Recovered sugar	Amount recovered	Total
	mg.	*microcuries*
Glucose	726	55
Fructose	925	30
Calcium-fructose complex, supernatant solution, calculated from reducing value	61	16.4
Calcium-fructose complex, wash water, calculated from reducing value	29	6.5
Total..................................	1741	107.9

The results indicated that the glucose crystallized from the mixture with labeled fructose contained 1.3 per cent fructose. The fructose was found to be contaminated with glucose to the extent of 2.5 per cent. The glucose was freed from the radioactive fructose contaminant almost completely by dissolving the sugar in water, adding inactive fructose, and recrystallizing. The glucose contaminant in the fructose was reduced to a negligible amount by dissolving the sample and oxidizing with barium hypoiodite solution as described by Goebel (8), passing the solution through ion exchange columns, and recovering the fructose by concentrating the solution and recrystallizing.

Preparation of Labeled Sucrose—For the production of sucrose a leaf from *Canna indica* was used. Leaves from this plant do not form any detectable starch but do produce a considerable amount of sucrose. Photosynthesis was carried out at 17°, since it was observed that the sucrose-reducing sugar ratio was more favorable at that temperature than at 28°. The ratio of sucrose to reducing sugars was 1.0 at 17°, whereas it was 0.7 at 28°.

A canna leaf with a blade from 12 to 15 cm. in length was picked from a new shoot on the rhizome, the petiole being cut to 2 cm. Such a leaf contains enough anthocyanin pigments nearly to mask the green color of the chlorophyll. Under the conditions of the experiment, when 500 mg. of barium carbonate were used at 17° with a 24 hour illumination period, all of the carbon dioxide was utilized. On a wet weight basis, the final level of reducing sugars was 1.4 per cent and that of sucrose 1.8 per cent.

The wet weight of the leaf, after it had been placed in the dark for 24 hours and then infiltrated with water through the petiole, was 4.92 gm. The leaf was placed in the photosynthetic chamber containing 227 microcuries of C^{14} in 2.5 mM of carbon dioxide, obtained from 0.5 gm. of barium carbonate. After 24 hours of illumination at 15°, the leaf was killed with alcohol as previously described and then refluxed overnight with 80 per cent alcohol.

The extract was concentrated on the steam bath, water being added from time to time to evaporate the alcohol, and the aqueous solution was extracted with ether in a separatory funnel. 200 mg. of sucrose carrier were added, the excess ether was evaporated on the steam bath, and the resultant aqueous solution passed over ion exchange columns. The neutral solution was concentrated to about 7 ml. and the reducing sugars fermented out with *Torula monosa.*

Torula monosa ferments glucose, fructose, and mannose, but does not attack sucrose or other disaccharides. The organism is grown aerobically for 24 hours at 28° on agar plates containing 0.5 per cent yeast extract and 1 per cent glucose. The cells are washed twice by centrifugation and resuspended in 0.033 M of phosphate buffer, pH 5. A yeast suspension prepared in this way will decompose approximately 15 mg. of glucose per hour per 100 mg. of dry yeast under anaerobic conditions at 37°. The initial glucose concentration should not be above 3 per cent.

The mixture of sucrose, yeast, and acid phosphate buffer was centrifuged and the supernatant sugar solution was again passed over the ion exchange columns after the addition of another 100 mg. of sucrose carrier. The neutral fraction was concentrated again and finally reduced to an immobile sirup in the vacuum oven. Upon the addition of warm absolute alcohol and stirring, the sucrose immediately crystallized. The crystals were placed in a desiccator and after 24 hours filtered off on a sintered glass funnel, washed with absolute alcohol, and dried with ether. The material was then ground in a mortar and assayed for radioactivity. The yield of sucrose was 360 mg., with a specific activity of 0.34 microcurie per mg., giving a total of 122 microcuries.

SUMMARY

Methods are described for the isolation of radioactive (C^{14}) starch, glucose, fructose, and sucrose from plants exposed to an atmosphere of radioactive carbon dioxide in the presence of light.

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THE IN VITRO SYNTHESIS OF HEME IN THE HUMAN RED BLOOD CELL OF SICKLE CELL ANEMIA*

Sirs:

Studies of the red blood cell in a human subject with sickle cell anemia have revealed a random disappearance of heme from the peripheral blood.[1] This finding is markedly different from the results obtained in similar studies in normal human subjects.[2] This difference may be due either to a random destruction of the red blood cells in sickle cell anemia or to a random synthesis and degradation of hemoglobin in the peripheral blood of the sickle cell anemia subject. In the investigation of this problem, the whole blood of subjects with sickle cell anemia was incubated with glycine labeled with N^{15}. Glycine has been shown to be the nitrogenous precursor of the protoporphyrin of hemoglobin.[2, 3] The heme isolated as hemin was

200 mg. of glycine labeled with 32 atom per cent excess N^{15} were incubated at 37° aerobically with 20 ml. of heparinized whole blood.

Experiment No.	Hematologic disorder	Reticulocytes	Time of incubation	N^{15} concentration in hemin N
		per cent	*hrs.*	*atom per cent excess*
1	Sickle cell anemia	15	24	0.050
2	" " "	17	24	0.071
3	" " "	20	24	0.071
4	" " "		24	0.077
5	Pernicious anemia*	21	18	0.011
6	Congenital hemolytic jaundice	14	24	0.015
7	" " "	16	24	0.007
8	" " "	11	24	0.013
9	Hypochromic anemia		24	0.020
10	Sickle cell trait		24	0.010
11–16	Normal controls (1 white, 5 Negro subjects)		24	0.000–0.015

* 100 mg. of isotopic glycine used.

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* Aided by a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council.

[1] Unpublished data from this laboratory.

[2] Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166**, 627 (1946).

[3] Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166**, 621 (1946).

found to contain a significant amount of N^{15}. These results demonstrate an *in vitro* synthesis of heme from glycine by the blood of sickle cell anemia subjects. This is in marked contrast to the findings in control studies with blood from normal subjects and from subjects with other hematologic disorders. These disorders include sickle cell trait without anemia, hypochromic anemia, and conditions, namely pernicious anemia and congenital hemolytic jaundice, associated with reticulocyte counts comparable to those characteristically found in sickle cell anemia.

These data demonstrate that there exists a mechanism in the blood of subjects with sickle cell anemia which can carry out the synthesis of heme *in vitro*. If this mechanism exists in the normal and in the other pathologic states studied so far, it is, under the conditions of these experiments, at best only slightly active. As yet no correlation has been made between the synthesis of heme and the components of sickle cell blood responsible for it. It appears not to be related to the mere presence of numerous reticulocytes.

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THE IN VITRO SYNTHESIS OF HEME FROM GLYCINE BY THE NUCLEATED RED BLOOD CELL*

Sirs:

It has been demonstrated, in both the rat and human,[1] that glycine is specificially utilized for the synthesis of protoporphyrin of hemoglobin. To facilitate the investigation of the mechanism of porphyrin formation a biological *in vitro* system was sought. Since hemoglobin in the non-nucleated mammalian red blood cell is normally produced prior to the loss of the nucleus, the ability of the nucleated red blood cell from the peripheral blood of the duck to synthesize heme *in vitro* was investigated. Incubation of red blood cells of the duck, either in whole blood or in saline, with glycine labeled with N^{15} leads to the formation of heme containing N^{15}. This demonstrates the utilization of glycine for the synthesis of heme *in vitro*. The data of typical experiments are given in the table.

200 mg. of glycine labeled with 32 atom per cent excess N^{15} were incubated at 37° aerobically with 20 ml. of heparinized duck blood and hemin subsequently isolated.

Duck No.	Time of incubation	N^{15} concentration of hemin N
	hrs.	
VP-8	24	0.126*
VP-1	12	0.109
VP-2	18	0.303
VP-3	4	0.051
VP-3	18	0.108
VP-3	24	0.113
VP-4	2	0.032
VP-4	6	0.051
VP-4	12	0.088
VP-9	24	0.090
VP-9	24†	0.006

* This hemin sample was converted to the dimethyl ester of protoporphyrin IX (Grinstein, M., *J. Biol. Chem.*, **167**, 515 (1947)). Its N^{15} concentration was 0.124 atom per cent excess N^{15}. $C_{36}H_{38}O_4N_4$, calculated N 9.49; found (Dumas) N 9.57.

† Incubated at 5°.

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* Aided by a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council.

[1] Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166**, 621, 627 (1946).

This system affords a means to investigate the mechanisms of the synthesis of heme. In addition the use of this *in vitro* system is being extended to the investigation of protein and nucleic acid synthesis.

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PHOSPHATES OF PYRIDOXAL AND PYRIDOXAMINE AS GROWTH FACTORS FOR LACTIC ACID BACTERIA*

Sirs:

Many cultures of lactic acid bacteria fail to grow in semisynthetic media containing all of the known B vitamins[1] even though oleic acid, recently shown to be essential for growth of many of these organisms,[2] is supplied in the medium.

*Comparative Effects of Malt, Pyridoxamine Phosphate, and Pyridoxal Phosphate on Growth of Lactobacillus helveticus**

Malt (aqueous extract)		Pyridoxal phosphate		Pyridoxamine phosphate	
	Turbidity†		Turbidity†		Turbidity†
mg. per 10 cc.		*γ per 10 cc.*		*γ per 10 cc.*	
0	95	0	95	0	95
2	87	0.01	78	0.003	81
3	78	0.05	47	0.005	51
4	69	0.10	44	0.01	45
5	57				
10	44				

* Incubation time, 24 hours at 37°. The basal medium was that previously described (foot-note 2), with yeast extract omitted, and with 50 mg. of acid-hydrolyzed, charcoal-treated casein, and 1 γ of pyridoxamine added per 10 cc.

† Per cent of incident light transmitted; distilled water = 100.

During fractionation of an unidentified factor required for growth of a strain of *Lactobacillus helveticus*, the activity of concentrates was found to be destroyed by light and by incubation with a crude preparation of malt phosphatase. This suggested that a phosphate of one of the light-labile vitamins might be concerned. Direct trial (see the table) showed that synthetic pyridoxal phosphate was highly active. Pyridoxamine phosphate, prepared from pyridoxal phosphate by transamination with glutamic acid,[3]

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[1] Rogosa, M., Tittsler, R. P., and Geib, D. S., *J. Bact.*, **54**, 13 (1947).

[2] Williams, W. L., Broquist, H. P., and Snell, E. E., *J. Biol. Chem.*, **170**, 619 (1947).

[3] Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, **169**, 643 (1947).

was 3 to 5 times as active as pyridoxal phosphate, and was 1 million times more active than malt, a natural source of the factor. Pyridoxal and pyridoxamine were present in the basal medium, and showed no activity for this organism. In contrast to the vitamin B_6 phosphates, flavin-adenine-dinucleotide and cocarboxylase showed no activity. A preparation of coenzyme II showed 0.003 the activity of pyridoxamine phosphate; coenzyme I was less active. Since these isolated preparations were only 20 and 40 per cent pure, respectively, their slight activity can probably be ascribed to contamination.

The requirement for phosphorylated pyridoxamine or pyridoxal may be quite wide-spread among lactic acid bacteria. A culture of *Lactobacillus acidophilus* also required these substances for growth. For this organism, pyridoxamine phosphate was about 6 times as active as pyridoxal phosphate, and again was 1 million times more active than malt. The amount of pyridoxamine phosphate required for maximum growth of these organisms is closely similar to the amount of pyridoxamine required by organisms able to use the unphosphorylated vitamin.[3]

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TRACER EXPERIMENTS ON THE MECHANISM OF GLYCINE FERMENTATION BY DIPLOCOCCUS GLYCINOPHILUS*

Sirs:

Glycine is decomposed anaerobically by *Diplococcus glycinophilus*[1] under appropriate conditions according to the equation $4CH_2NH_2COOH + 2H_2O \rightarrow 4NH_3 + 3CH_3COOH + 2CO_2$. The mechanism of this fermentation has been investigated by the use of C^{14}-labeled glycine, carbon dioxide, and acetic acid. The results of several experiments are summarized in the table.

The data support the following conclusions. (1) Approximately 75 per cent of the methyl carbon and 54 per cent of the carboxyl carbon of acetic acid are derived from the methylene carbon of glycine (Experiment 1). (2) From 90 to 95 per cent of the carbon dioxide is derived from the carboxyl carbon of glycine, the remainder coming from the methylene carbon (Experiments 1 and 2). These observations indicate that one of the main reactions in the fermentation is a condensation between 2 molecules of glycine, or derivatives therof, through their methylene groups. The terminal carbon atoms of the resulting compound, possibly a C_4-dicarboxylic acid, are converted mainly to carbon dioxide, and the 2 central carbon atoms are oxidized to acetic acid. The low specific activity of the carbon dioxide

Glycine Fermentations with C^{14}-Labeled Substrates

Experiment No.	Substrates*	Specific activity, counts per min. per mM				Per cent recovery of C^{14}
		Labeled substrate	Products			
			CO_2	HAc-CH_3	HAc-COOH	
1	$C^*H_2NH_2COOH + NaHCO_3$	12,600	160	9,400	6,800	91
2	$CH_2NH_2C^*OOH$	17,200	15,500	2,450	8,200	96
3	$CH_2NH_2COOH + NaHC^*O_3$	27,600	14,500	1,260	7,460	99
4	$CH_2NH_2COOH + CH_3C^*OONa$	1.5×10^6	180	150	18,200	116
5	$CH_2NH_2COOH + C^*H_3COONa$	4.5×10^6	85	12,100	160	70

* Washed cell suspensions of *D. glycinophilus* were allowed to act under anaerobic conditions for 16 to 20 hours at 37° on a medium containing approximately 0.04 M glycine, 0.02 M phosphate buffer, pH 7.0, and 0.02 per cent $Na_2S \cdot 9H_2O$. When bicarbonate was added, the initial concentration was from 0.11 to 0.15 M. Acetate was added to Experiments 4 and 5 in tracer amounts ($<5 \times 10^{-4}$ M).

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* Supported in part by a research grant from the United States Public Health Service.

[1] Cardon, B. P., and Barker, H. A., *Arch. Biochem.*, **12**, 165 (1947).

formed from methylene-labeled glycine (Experiment 1) shows that a complete oxidation of glycine can occur only to a small extent. (3) At least 6 per cent of the methyl carbon and 38 per cent of the carboxyl carbon of acetate originate from carbon dioxide (Experiment 3). A comparison of the specific activities of the acetate carboxyl carbon in Experiments 2 and 3 indicates that a direct reduction of glycine to acetic acid is not an important reaction. (4) Acetate is metabolized very slowly if at all (Experiments 4 and 5). This eliminates the possibility that carbon dioxide fixation in this organism involves the reactions $CH_3COOH \xrightarrow{+CO_2} CH_3COCOOH \xrightarrow{+CO_2} COOHCH_2COCOOH \xrightarrow{+6H} 2CH_3COOH$ which would result in a redistribution of the labeled carbon in acetic acid.

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Received for publication, February 24, 1948

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THE RÔLE OF PANTOTHENIC ACID IN THE METABOLISM OF PYRUVATE BY PROTEUS MORGANII

Sirs:

It has been shown by Dorfman, Berkman, and Koser[1] and by Hills[2] that pantothenic acid is concerned in the oxidation of pyruvate by *Proteus morganii*. This conclusion was based on the stimulation of oxygen uptake by added pantothenate when cells grown in a medium deficient in pantothenic acid were used.

Recently, Novelli and Lipmann[3] have shown that this stimulation is paralleled by an increase in concentration of coenzyme A. Since coenzyme A is necessary for the acetylation of sulfanilamide, these authors have suggested that the pantothenate effect is concerned with the primary attack on acetate.[4]

Experiment No.	No pantothenate			0.1 mg. Ca pantothenate		
	O_2	CO_2	R.Q.	O_2	CO_2	R.Q.
	microliters	*microliters*		*microliters*	*microliters*	
1	25	134	5.4	217	600	2.8
2	46	158	3.5	262	386	1.5
3	46	147	3.1	249	508	2.0
Average..	39	146	3.7	243	498	2.0

Balance studies performed in this laboratory indicate that the stimulation by pantothenic acid of oxygen uptake of *Proteus morganii* with pyruvate as substrate cannot be accounted for by oxidation of the acetate. The table shows the relation between the oxygen absorption and CO_2 output of deficient cells with and without added pantothenate.

The high R.Q. of the unstimulated reaction indicates that a decarboxylation takes place but that the oxidation to acetate depends upon an adequate supply of pantothenic acid.

Further investigation has shown that a compound giving a positive Voges-Proskauer reaction accumulates in the reaction mixture when pantothenate has not been added. This compound has been identified as acetylmethylcarbinol by the formation of nickel dimethylglyoxime after oxidation with ferric chloride.

---

[1] Dorfman, A., Berkman, S., and Koser, S. A., *J. Biol. Chem.*, **144**, 393 (1942).

[2] Hills, G. M., *Biochem. J.*, **37**, 418 (1943).

[3] Novelli, G. D., and Lipmann, F., *Arch. Biochem.*, **14**, 23 (1947).

[4] Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **171**, 833 (1947).

The high R.Q. and the accumulation of acetylmethylcarbinol in the unstimulated reaction are not easily explained if the primary attack of the pantothenate enzyme is an acetate. These data suggest that a pantothenic acid containing coenzyme is concerned with the utilization of acetylmethylcarbinol or some closely related substance.

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REVERSIBLE INHIBITION OF THE COUPLING BETWEEN PHOSPHORYLATION AND OXIDATION

Sirs:

Clifton[1] was among the first to show that dinitrophenol (DNP) in low concentrations completely blocked synthetic reactions without interfering with oxidation. Other workers have shown that this drug inhibits nitrogen assimilation,[2] growth and differentiation,[3] the formation of adaptive enzymes,[4] and Hotchkiss[5] has reported preliminary data showing that DNP prevents phosphate uptake by respiring yeast cells. These results would appear to indicate that DNP acts on the basic mechanism in the cell by which phosphate bond generation is coupled to oxidative reactions.

TABLE I

All samples contained 1.0 cc. of an enzyme preparation similar to that of Green *et al.*,[6] prepared by centrifuging a rabbit kidney homogenate in KCl-$NaHCO_3$ buffer and washing the residue twice with fresh buffer. To this was added 0.1 cc. of yeast hexokinase, 0.0067 M $MgCl_2$, 0.013 M NaF, 0.00067 M adenosine-5-phosphate, 0.02 M phosphate buffer of pH 7.2, 0.0167 M fructose, and 0.01 M Na glutamate as substrate. Identical control cups were prepared, into which acid from a side arm was tipped at the beginning of the experiment to provide the initial level of inorganic phosphate. Temperature, 25°; gas phase, air; time, 6 minutes.

Additions	Oxygen uptake	Phosphate uptake	P:O ratio
	microatoms	*micromoles*	
None	8.0	17.5	2.2
1.8×10^{-4} M DNP	7.9	1.3	0.2

During a study of this coupling mechanism, it was observed that 5×10^{-5} to 2×10^{-4} M DNP prevented phosphorylation without affecting or with slightly stimulating oxidation.

Concentrations of DNP as low as 5×10^{-6} M were found to lower markedly the P:O ratio, an effect that could be reversed by washing out the DNP with fresh buffer. Furthermore, it was found that DNP could "replace" inorganic phosphate, which otherwise is a compulsory component of this system. It appears that the phosphate-deficient system is strongly stimulated by DNP, while the complete system responds only with a slight stimulation (see Table II).

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[1] Clifton, C. E., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, 6, 269 (1946).

[2] Winzler, R. J., Burk, D., and du Vigneaud, V., *Arch. Biochem.*, 5, 25 (1944).

[3] Clowes, G. H. A., and Krahl, M. E., *J. Gen. Physiol.*, 20, 145 (1936).

[4] Spiegelman, S., *J. Cell. and Comp. Physiol.*, 30, 315 (1947).

[5] Hotchkiss, R. D., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, 4, 153 (1944).

[6] Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, 172, 389 (1948).

These results indicate that DNP reversibly uncouples phosphorylation from oxidation, an effect that can also be obtained with atebrin (mepacrine) in 10^{-3} M concentration. Although sodium azide can lower the P:O ratio, it cannot replace phosphate in the system and is, in slightly higher concentration, a powerful inhibitor of respiration as well. DNP does not inhibit respiration except in high concentration.

TABLE II

Temperature, 25°; gas phase, air; time, 30 minutes.

Phosphate, M	0	0	2×10^{-2}	2×10^{-2}
DNP, M	0	8×10^{-5}	0	8×10^{-5}
O_2, *micromoles*	5.1	17.9	18.6	21.0

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Received for publication, February 13, 1948

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GLYCINE CONTENT OF DL-ALANINE

Sirs:

In attempts to employ the assay method for glycine,[1] consistently high blanks were obtained which vitiated assay results. Subsequent receipt of a relatively pure grade of DL-alanine permitted the determination of the amount of glycine in all of our amino acids. Only DL-alanine and arginine yielded results which suggested that they were contaminated with glycine. However, the presence of glycine in arginine seems to be apparent rather than real; this effect is being investigated in this laboratory.

Glycine Content of Alanine

Lot No.	Source of DL-alanine	Mean per cent glycine
1	A	0.20
2	", recrystallized 3 times	0.06*
3	" " 6 "	0.04*
4	" " 9 "	0.03*
5	B, Lot 1	1.05
6	" " 2	0.39
7	" " 3	0.39
8	" " 4	0.16
9	" " 5	0.99
10	" " 6	1.86
11	C	0.21
12	D	0.35
13	E	2.7
14	D, β-alanine	<0.05

* Approximate.

In the glycine assay,[1] 8 mg. of DL-alanine are employed per 2 ml. of total volume. The induction period permits 6 γ or 0.08 per cent of glycine in the alanine without increase in blank titration. This amount may be further increased by the use of a more dilute inoculum, a practice which the authors[1] do not recommend. We report herewith the glycine content of DL-alanines commercially available (see the table).

Ten commercial lots of DL-alanine, three lots recrystallized three, six, and nine times, respectively, and one lot of β-alanine were assayed by the microbiologic method[1] for their glycine content. Five assay levels were used per sample. Decreasing assay values with increasing amount of DL-alanine were found consistently when the top assay level contained more than 4 mg. of added DL-alanine per 2 ml. of final volume. This decrease is approximately linear with added DL-alanine. Since the value reported is a mean value, it represents a minimal amount of glycine in these samples.

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[1] Shankman, S., Camien, M. N., and Dunn, M. S., *J. Biol. Chem.*, **168**, 51 (1947).

Henderson and Snell[2] have reported a medium containing 0.4 mg. of DL-alanine per 2 ml. of final volume in which they find one recrystallization of alanine satisfactory. At this level of alanine only 4 γ of glycine are introduced at 1 per cent glycine impurity level. Whether this is significant is not possible to say with the brief description given.[2]

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[2] Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).

THE EFFECT OF PYRUVATE AND INSULIN ON FATTY ACID SYNTHESIS IN VITRO*

Sirs:

The utilization of acetic acid for the synthesis of both cholesterol and fatty acids by intact animals has been demonstrated with the aid of labeled acetate.[1] In rat liver slices acetate carbon is readily incorporated into cholesterol, but under these conditions the uptake of isotope by the higher fatty acids is very small.[2] In order to investigate further the *in vitro* synthesis of fatty acids, rat liver slices were incubated in Krebs-bicarbonate buffer which contained, in addition to $CH_3C^{14}OONa$, one of the following non-isotopic compounds: glucose, pyruvate, oxalacetate, fumarate, malate, or succinate. The incorporation of acetate carbon into liver fatty acids was increased several fold by pyruvate and to a somewhat smaller extent by oxalacetate or glucose. The other dicarboxylic acids were ineffective.

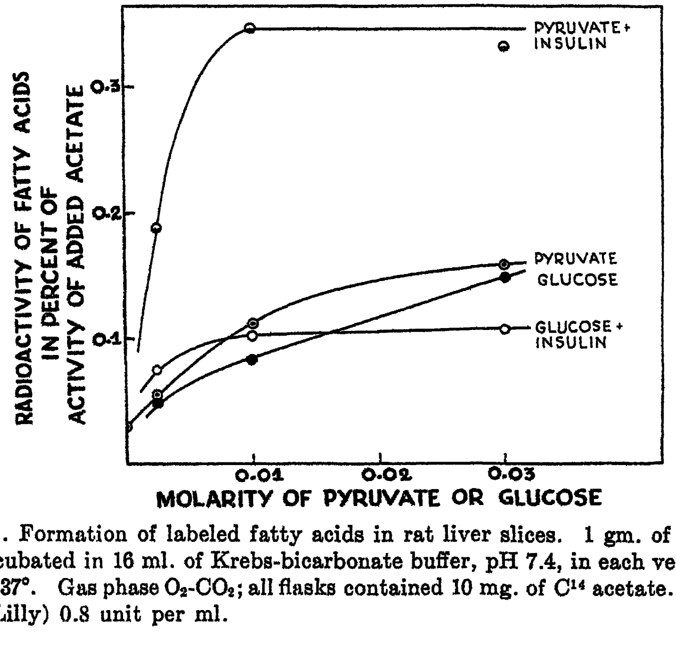

FIG. 1. Formation of labeled fatty acids in rat liver slices. 1 gm. of rat liver slices incubated in 16 ml. of Krebs-bicarbonate buffer, pH 7.4, in each vessel for 3 hours at 37°. Gas phase O_2-CO_2; all flasks contained 10 mg. of C^{14} acetate. Insulin (iletin, Lilly) 0.8 unit per ml.

* Aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[1] Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **143**, 297 (1942). Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **154**, 311 (1944).

[2] Bloch, K., Borek, E., and Rittenberg, D., *J. Biol. Chem.*, **162**, 441 (1946).

Addition of insulin to the medium afforded a further increase of fatty acid synthesis when the medium contained pyruvate but had no additional effect when glucose was present (see the graph). When acetate was the only substrate, insulin depressed the uptake of isotope by the fatty acids. The stimulatory effect was shown by both crystalline zinc insulin and by an amorphous preparation. A rôle for insulin in the conversion of carbohydrate to fat has been suggested by Drury[3] and emphasized by Stetten and Klein[4] who observed a depressed rate of fatty acid turnover in rats treated with alloxan. The data presented here suggest that one of the actions of insulin is concerned with the metabolism of pyruvate in general[5] and specifically with the utilization of pyruvate for the synthesis of fatty acids.

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Received for publication, February 18, 1948

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[3] Drury, D. R., *Am. J. Physiol.*, **131**, 536 (1940).
[4] Stetten, D., and Klein, B., *J. Biol. Chem.*, **159**, 593 (1945).
[5] Rice, L., and Evans, E. A., Jr., *Science*, **97**, 470 (1943).

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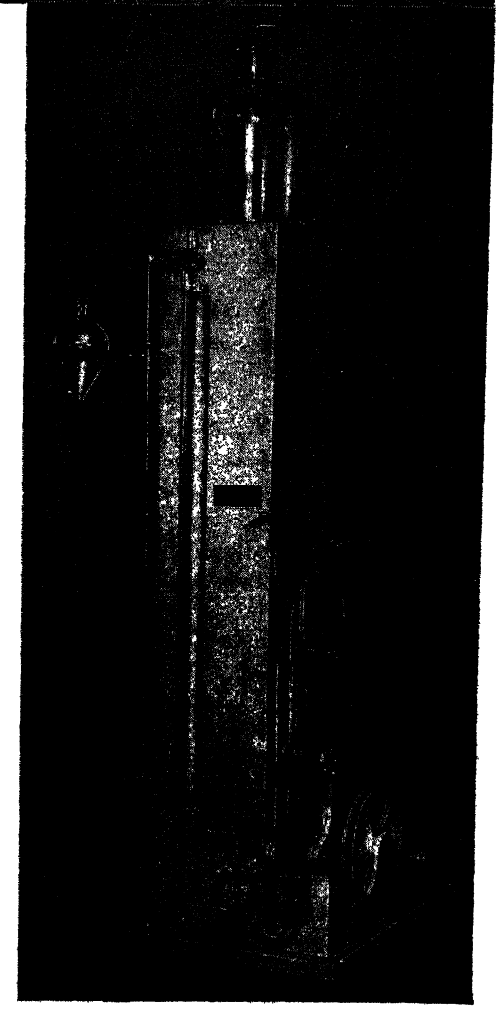

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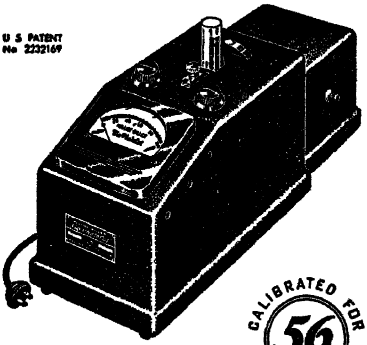

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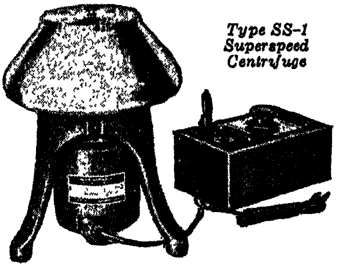

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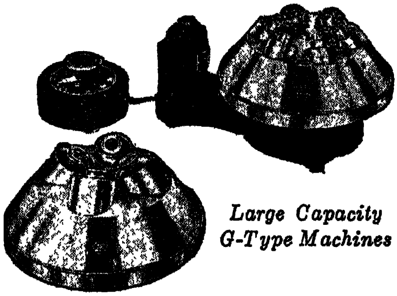

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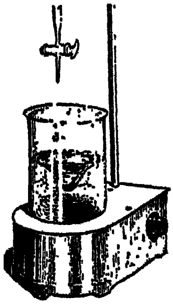

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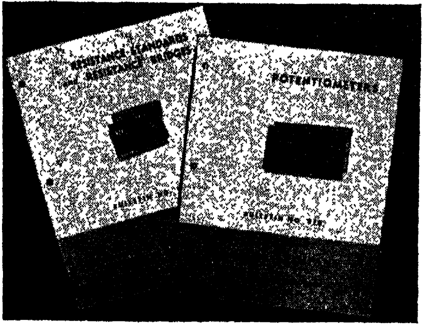

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